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(54) Title: MATERIALS AND METHODS FOR PRODUCING DNA LIBRARIES AND DETECTING AND IDENTIFYING MICROORGANISMS

(57) Abstract: The present invention concerns materials and methods for producing virtual genomic libraries of microorganisms, such as viruses. The genomic libraries produced are useful for rapidly mapping genetic traits, such as those conferring increased pathogenicity or resistance to antiviral or antibiotic drugs, and as diagnostic tools for distinguishing from among a family of bacteria or viruses, such as orthopoxviruses. In one aspect, the present invention pertains to methods for synthesizing entire viral genomes. In another aspect, the present invention pertains to a diagnostic method for identifying an unknown virus using a plurality of polymerase chain reaction (PCR) primers (primer sets). In yet another aspect, the present invention concerns a method for designing PCR primers and primer sets useful in carrying out the diagnostic method disclosed herein. In still another aspect, the present invention concerns PCR primers that have been produced using the primer design method disclosed herein. In another aspect, the present invention concerns diagnostic kits for determining the identity of one or more unknown microorganisms.

DESCRIPTION

MATERIALS AND METHODS FOR PRODUCING DNA LIBRARIES AND DETECTING AND IDENTIFYING MICROORGANISMS

Cross-Reference to Related Application

The present application claims the benefit of U.S. Provisional Application No. 60/462,204, filed April 11, 2003, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

Government Support

The subject matter of this application has been supported by a research grant from the National Institutes of Health under grant number AI 15722. Accordingly, the government may have certain rights in this invention.

Background of the Invention

Core to the field of microbiology is the ability to positively identify microorganisms at the level of genus, species, or serotype. Correct identification of microorganisms is an essential tool in the laboratory and clinical environment, and plays an important role in the control of microbial contamination in the processing of foodstuffs, the production of agricultural products, and the monitoring of environmental media such as ground water. Of greatest concern is the detection and control of pathogenic microorganisms.

Although a broad range of microorganisms has been classified as pathogenic, recent interest in pathogenic microorganisms is related to their potential as a bioterrorism agent. Therefore, recent attention has primarily focused on the bacterial and viral causative agents of anthrax (*Bacillus anthracis*), plague (*Yersinia pestis*), botulism (*Clostridium botulinum*), cholera (*Vibrio cholerae*), viral hemorrhagic diseases (Ebola virus, Marburg virus, flavivirus), and smallpox (Variola virus (orthopox)), and new variants of such agents.

The genus *Orthopoxvirus* of the family *Poxviridae* includes at least 12 known nine species, four of which are able to cause human diseases of various severities. The most pathogenic member of this taxonomic group is variola virus, which has been considered eradicated as a natural cause of smallpox since 1977. Other orthopoxviruses (OPVs), such as cowpox virus, buffalopox virus (a subspecies of vaccinia virus), and monkeypox virus, can also infect humans through contact with animals.

Identification among orthopoxvirus species and strains has been achieved by various immunologic and biologic methods, including virus neutralization, hemagglutination inhibition and other serologic assays, determination of plaque or pock morphology, reproductive ceiling temperature in cello cultures or on chicken embryo chorioallantoic membranes, lethality or infectivity for various animals or selected tissues of animals, the ability of infected cells to hemadsorb or hemagglutinate chicken erythrocytes, analysis of virus proteins by polyacrylamide gel electrophoresis, determinations of genome DNA endonuclease cleavage profiles, determination of DNA restriction maps, determination of nucleotide sequences, and various PCR methods (Lappa, S. *et al.*, *J. Clinical Microbiology*, 40(3):753-757, March, 2002; Ropp, S. *et al.*, *J. Clinical Microbiology*, 33(8):2069-2076, August, 1995).

Virus mutants exhibiting various phenotypic properties, such as resistance to antiviral drugs, are easily generated both in nature and in the laboratory. Since the early 1970s, "marker rescue" has been used for several DNA viruses, such as Adenoviruses, Herpesviruses, Polyomaviruses, Poxviruses, and bacteriophages, as a method for the physical mapping of genome fragments and mutations. Currently, the viral DNA fragments used for such mapping have consisted of restriction enzyme-generated fragments of various sizes, and other conventional sources of DNA. Mapping methods involve cloning to prepare genomic libraries, using plasmid or cosmid vectors. (Hutchison, C.A., III & Edgell, M.A., *J. Virol.*, 8:181-189, 1971; Arrand, J. E., *J. Gen Virol.*, 41:573-586, 1978; Frost, E. & Williams, J., *Virology*, 91:39-50, 1978; Wilkie, N.M. *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 39:657-666, 1974; Miller, L.K. & Fried, M., *J. Virol.*, 18:824-832, 1976; Nakano, E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:1593-1596, March 1982; Weisbeek, P.J. *et al.*, *Virology*, 72:62-71, 1976); Lai, C.J. & Nathans, D., *Virology*, 60:466-475, 1974.

A major hurdle in the mapping of mutations, particularly for the larger DNA viruses, has been the construction of genomic libraries, which is very time consuming. This older, classical approach requires the isolation and preparation of cosmid/plasmid clones, from the mutant in question followed by use of the library in "marker rescue" to map the mutation of interest. Each mutant requires preparation of a new genomic library, which is a very time consuming procedure.

Briefly, if the entire genome of a virus is broken up into fragments with restriction enzymes, the fragments can be separated and maintained as individual fragments of DNA, usually by cloning them into a vector or artificial chromosome, such as a cosmid. If successful, a genomic library is created of the virus. Genomic libraries are generated from mutant viruses in order to locate the mutation that they carry. Usually, the mutation the researcher is interested in finding gives the virus a significant growth advantage under certain conditions, such as the ability to replicate in the presence of antiviral drugs. Poxviruses are some of the largest DNA viruses (containing approximately two hundred thousand bases), and due to the size of their genome, the researcher usually resorts to making genomic libraries by cloning large DNA fragments from the genome into cosmid vectors (large circular DNAs that can be cloned and replicated). Cosmid library construction is labor intensive, expensive, and requires a great deal of time.

Currently available PCR library construction kits (STRATAGENE) are utilized for a completely different objective. Such kits are designed to make cDNA libraries that represent the current repertoire of mRNA during the time of RNA isolation. This approach would not be useful for libraries of viral DNA, for example. Such kits are only necessary for higher eukaryotes (mouse, human, *etc.*), which contain several introns of non-coding DNA within a given gene. Isolation of strictly viral sequence information in the library would be nearly impossible. Another disadvantage is that these libraries only contain the DNA that actually encodes proteins, with no non-coding sequences. Therefore, these libraries are not useful for detecting "non-coding" mutations, such as those found in gene promoters. Furthermore, each segment within these libraries is "weighted" with respect to the number of mRNA molecules that were present in the original sample during RNA isolation. Therefore, less abundant mRNA transcripts (which may contain the mutation being screened for) would not be detectable in any

subsequent marker rescue experiments required for genetic mapping in cells infected with poxviruses, for example.

Thus, due to the high cost and labor involved in using cosmid library reagents, construction of genomic libraries and physical mapping of unique mutations has been somewhat limited to only a handful of viruses at a time for any given laboratory.

Brief Summary of the Invention

The present invention concerns materials and methods for producing virtual genomic libraries of microorganisms, such as bacteria and viruses. The genomic libraries produced are useful for rapidly mapping genetic traits, such as those conferring increased pathogenicity or resistance to antiviral or antibiotic drugs, and as diagnostic tools for distinguishing from among a family of bacteria or viruses, such as orthopoxviruses. In one aspect, the present invention pertains to methods for synthesizing entire viral genomes.

In another aspect, the present invention pertains to a diagnostic method for determining whether a target microorganism is present within a biological or environmental sample and, optionally, identifying the microorganism within a group of genetically related microorganisms, using a plurality of polymerase chain reaction (PCR) primers (primer sets). In one embodiment, the method is used to determine whether an unknown virus present in a sample is a poxvirus. In another embodiment, the method is used to determine the identity of an orthopoxvirus present within a sample. The diagnostic method of the present invention is particularly useful for determining the identity (*e.g.*, genus and species) of the microbial agent responsible for an infection in a patient.

In yet another aspect, the present invention concerns a method for designing PCR primers and primer sets useful in carrying out the diagnostic method disclosed herein. In still another aspect, the present invention concerns PCR primers that have been produced using the primer design method disclosed herein, and PCR products of those primers.

In another aspect, the present invention concerns diagnostic kits for determining the identity of one or more unknown microorganisms present in a biological or environmental sample. In one embodiment, the diagnostic kit contains 4 to 7 PCR primer pairs.

The present inventors have developed a series of 42 PCR primer pairs with a single, common annealing temperature to generate overlapping 5kb DNA fragments that span the entire 198kb rabbitpox virus genome. Thus, in a single set of PCR reactions, it is possible to synthesize a high-resolution virtual genomic library for any given rabbitpox isolate (wild type or mutant), from a single stock of 84 oligonucleotides and commercially available DNA polymerase (such as VENT DNA polymerase).

Since most of the PCR primers were derived from the vaccinia virus genomic sequence, a virus with high homology to rabbitpox virus, this approach has been extended to develop a similar library for vaccinia virus. Data based on homology with genomic sequence data shows that these PCR primers may be used in other species of orthopoxviruses, such as smallpox (variola virus), and monkeypox viruses, with very little or no modification.

Advantageously, the fact that all rabbitpox virus primers do not all work with other closely related viruses provides a method of the subject invention, which is a rapid, differential diagnostic method by which to distinguish closely related but not identical viruses. For example, in one embodiment, the diagnostic method of the present invention can be used to determine if a subject is suffering from a smallpox infection. The use of this method for creating libraries of other DNA viruses, or any other similarly sized genome (bacterial or eukaryotic), would also be of consistent interest in mapping mutations, manipulation, and differential diagnosis of an infection in a research or clinical setting.

Since the ability for a virus to gain resistance to antiviral drugs is due strictly to genetic mutations in the virus, the mutated gene or genes responsible for this resistance can be identified by viral genetic techniques. As a result, the proteins encoded by these drug resistance genes can also be identified, and the function of these proteins can be studied, if currently unknown. Understanding how a given gene or its gene product contributes to drug resistance will allow for better, more specific drugs to be developed that can target the new drug resistance genes that have been identified with this approach, making the virus more susceptible to antiviral drug therapy. Attenuation of any potential virulence or toxicity caused by vaccinations with clinical strains of live virus is also a desirable goal. The simplicity of making PCR libraries using the method of the present

invention would facilitate the development of viruses containing modifications in (or removal of) genes identified by methods outlined herein.

The ability of a virus to grow in different animal species is a genetic characteristic referred to as its host range. Changes in host range, such as a virus acquiring the ability to infect humans through genetic mutation, can also be studied using the methods of the present invention. For example, if an undesirable viral infection of humans could (hypothetically) have come from a virus that originated in birds, then understanding and counteracting the mechanisms involved in this change in the virus's host range could also be achieved by genetic mapping and identification of mutations. The PCR library method of the present invention can be utilized in conjunction with existing marker rescue techniques to facilitate the identification of these mutations in several strains at once, with very little cost or effort.

The diagnostic method of the present invention can be used (*e.g.*, by medical technicians) to positively identify or rule out a particular microorganism as an infectious agent. For example, the medical technician can determine if a subject's unknown infection is from an orthopoxvirus and, if so, which orthopoxvirus. The subject may be presenting various non-specific symptoms, such as fever and sores on the skin, for example. The rapid and accurate detection of a smallpox infection (variola virus) would allow for intervention of an appropriate therapy specific to smallpox, which would likely prevent any death or disease from the infection. The diagnostic method of the present invention can be utilized as a superior alternative to conventional histopathological examination methods, or used in conjunction with such methods. The methods of the present invention can be carried out in a very rapid manner, permitting the clinician to avoid unneeded or overly broad treatments or prophylactic interventions. The primers and diagnostic method of the present invention can be used to identify one or more unknown microorganisms from among a wide variety of microorganisms, including pathogenic microorganisms, such as pathogenic viruses and bacteria. For example, the primers and method of the present invention can be used to identify unknown microorganisms from among those described in the Morbidity and Mortality Weekly Report (Center for Disease Control, October 19, 1990, Volume 39, No. RR-13), which is incorporated by reference herein in its entirety.

The methods of the present invention can be automated in a vessel-based system, thereby reducing risk of human error and contamination. In an automated system, reagents for the various reactions are added sequentially according to a programmed sequence.

Other uses for the PCR libraries of the present invention are the same as those of conventional DNA libraries in other biological systems. However, the present invention provides significant advantages in saving considerable time and resources, as well as providing a very high-resolution power due to the small size of each segment in the PCR library, which is not strictly limited by any vector-cloning restraints.

Brief Description of the Drawings

Figure 1 shows a conceptual layout of 42 PCR products present in the rabbitpox virus (RPV) PCR library of the subject invention. Each product is 5kb in length and can be generated when using the primer pairs shown and viral DNA template. For example, 5kb product #1 is made by pairing "IDT3" (SEQ ID NO:1) and "IDT4" (SEQ ID NO:2) primers in a single PCR, and so forth. Similarly, a 15kb PCR library can be produced by pairing primers indicated in bold. For example, IDT3 can be paired with IDT9, FS396 with IDT13, etc. Also shown is a linear representation of the 198kb template. Digestion of the RPV genome with the Hind III restriction enzyme generates the fragments indicated, which are ordered alphabetically by size, "A" being the largest. The position of each PCR product shown in the upper portion of Figure 1 closely represents its actual location on the physical genomic map shown in the lower portion of Figure 1.

Figures 2A and 2B show 5kb PCR libraries for rabbitpox (RPV) and vaccinia (VVwr) viruses. Virtually the entire RPV and VVwr virus genomes were divided into 42 and 40 overlapping PCR products, respectively, omitting only non-coding sequence at each terminus. Each of the products shown (5µl PCR product per lane) average 5kb in length and was generated using a different PCR primer pair for each reaction.

Figure 2C shows each RPV PCR product (approximately 5kb each) drawn to scale in boxes above HindIII restriction maps for RPV and VVwr. Products designed for the VVwr library were given the prefix "V" for distinction from RPV products and indicated above the RPV PCR product map with a line.

Figure 3 shows representative data generated using the two-step marker rescue technique. The marker rescued in this case is the ability for virus to replicate on Human Lung cells (A549).

Figures 4A and 4B show preliminary data obtained from rabbitpox virus (RPV) DNA and cowpox virus (CPV) DNA, respectively. These experiments were repeated with more purified CPV template at a lower T_m, with results shown in Figure 9.

Figures 5-8 show PCR libraries for vaccinia virus (WR), monkeypox virus, camelpox virus, and cowpox virus, respectively, for comparison. All gels have the same layout and labeling, using the same primer sets for all reactions. The primer stocks that failed for vaccinia virus (see lack of PCR product in lanes for primers V7, 7, and 10, for example, in Figure 5) in this series of experiments were later replaced and verified in subsequent experiments, as shown in Figure 10.

Figure 9A shows the predicted and observed sizes (in kb) of PCR products using the VVwr PCR library primers and DNA from different species of orthopoxviruses. Only three mismatches of the primer sequence against the DNA template were allowed in this analysis. Predicted sizes in boldface should distinguish each species of virus. Vaccina virus (WR) serves as the positive control by which others are compared.

Figure 9B-9D shows the detection and differentiation of different species of orthopoxviruses. Shown are PCR products obtained from the combination of VVwr PCR library primers and DNA from different orthopoxviruses. This demonstrates the size of each product present in kilobases. In camelpox virus (Figure 9C), products #36 and #39 are absent and the size of product #37 is different from product #37 of WR. Similarly, product #39 of cowpox virus (Figure 9D) is different in size from product #39 of WR. Each of these changes was predicted as reported in Figure 9A.

Figure 10 shows verification that the new, revitalized primer stocks work on purified rabbitpox virus genomic DNA template.

Figures 11A-11D show 5kb PCR libraries of P14+ wt D5R #1, P14+ wtD5R #2, P14+ wtD5R #3, and ΔSPI-1+ E9ΔH142 #1, respectively, which are independently isolated RPV mutants. The PCR libraries for these mutants were synthesized in a single afternoon.

Figure 12 shows an amino acid alignment of the reactive center loop of inhibitory serpins demonstrating conservation of threonine (T) at the "P14" positioning the C-

terminal reactive site loop (RSL). Naturally, non-inhibitory serpins ovalbumin and angiotensinogen (Angiotens.) have an arginine (R) at this site. The "P1" residue confers specificity of each serpin to its target preoteinases listed on the right. Targeted proteolysis cleaves the bond between P1-P1' residues. Pox = poxvirus serpins. Hum = human serpins. RPV = rabbitpox virus. CPV = cowpox virus. α1-Antichy = human alpha 1 antichymotrypsin. α1-Antitryp = human alpha 1 antitrypsin. Cat G = human cathepsin G. ICE = human interleukin-1 β converting enzyme. Granz B = human granzyme B. tPA = human tissue-type plasminogen activator.

Figures 13A-13C show the results of SDS-polyacrylamide gel mobility shift assays. Protease inhibitory function is estimated by the ability of 35S-labeled SPI-1 protein synthesized *in vitro* (TNT system, PROMEGA) to form highly stable high molecular weight complexes with increasing amounts of purified Cathepsin G. These complexes are visualized in an SDS-polyacrylamide gel mobility shift assay. ES* represents the most stable SPI-1/Cathepsin G bimolecular complex, that is supershifted by ~25kDa relative to uncleaved SPI-1 (45kDa). The host range of each virus with either a wild type or a mutant SPI-1 gene and the corresponding activity of each SPI-1 protein *in vitro* are shown in Figures 13A-13C. Figure 13A shows that wild type SPI-1 protein is able to form a stable ES* complex with Cathepsin G. Rabbitpox virus (RPVwt) has a broad host range in cell culture, as shown by the ability to form viral plaques on A549 cells. Figure 13B shows that the "P14" mutant (T309R) SPI-1 protein has lost the ability to form an ES* complex with Cathepsin G. The RPV SPI-1 T309R mutant has a reduced host range, as shown by the inability to form viral plaques on A549 cells (non-permissive). Figure 13C shows selection for phenotypic revertants of RPV SPI-1 T309R that can form viral plaques on A549 cells, yielding an intragenic suppressor with a reversion to cysteine at the P14 position to restore protease inhibitory function. This demonstrates the only known example of an inhibitory serpin with a cysteine at the P14 position.

Figure 14 shows a procedure carried out to map temperature sensitive extragenic suppressors. Figure 14 shows that one extragenic suppressor of the SPI-1 T309R mutation created a temperature sensitive (*ts*) mutant virus. This phenotype was used to map the extragenic suppressor by a *ts* marker rescue protocol. Recombinant viruses were generated by transfection of infected cells with wild type (wt) DNA fragments. Viruses

that have been rescued for the *ts* phenotype by recombination with wt DNA at the *ts* allele are selected at 40° C and detected in a viral plaque assay.

Figure 15 shows a genomic cosmid library and Hind III map of vaccinia virus (WR strain). Eleven overlapping cosmids are shown with respect to the Hind III restriction map of the 195 kb VV-WR genome (Stanley, P., *et al.*, *Biochemistry*, 42:6241-6248). Hind III fragments are labeled alphabetically by decreasing size. Cosmids #3 and #4 (but not #2) provided rescue to an RPV *ts* mutant (Sup 1), which mapped the mutation to within 10 kb (shown by dotted lines) between the Hind III E and I fragments.

Figure 16 shows TS marker rescue of RPV Sup1 with wtPCR products. CV 1 cells were infected at 41°C under agarose medium with the *ts* mutant RPV Sup1 and transfected with PCR products from wild type virus DNA representing the entire open reading frames E8R, E9L, E10R, and SPI 1. *ts*E9 is E9L from the Sup1 mutant DNA template. Mock = uninfected cells. The open reading frames used are shown to scale and in order along the RPV genome. The Sup1 mutation was later identified within E9L by DNA sequencing.

Figure 17 shows plated CV-1 cells or A549 cells infected with equal dilutions of each virus at 37 °C or 40 °C, and corresponding RPV genome for each mutant, demonstrating that the *ts* mutant viral DNA polymerase is a SPI-1 T309R suppressor. Row A (RPVwt) shows that wild type virus forms plaques on A549 and CV-1 cells, regardless of the incubation temperature. Row B (SPI-1 T309R) shows that the "P14" mutant of SPI-1 (T309R), created by site-directed mutagenesis (Moon, K *et al.*, *J. Virol.*, 1999, 73(11):8999-9010), reduces viral host range, but does not make the virus temperature sensitive (*ts*), shown by viral plaques on CV-1 cells at 40° C. Row C (SPI-1 T309R/DNA PolΔH142) shows one result from a selection for phenotypic revertants on A549 cells at 37° C, which yielded one *ts* mutant virus, with a mutation extragenic to the SPI-1 gene that mapped to the viral DNA polymerase (DNA PolΔH142) by marker rescue (shown in Figure 16). In Row D (SPI-1 T309R reconstructed), genetic linkage of the *ts* phenotype to extragenic suppression was established when the virus rescued for *ts* (SPI-1 T309R reconstructed) by a wild type DNA pol gene (E9L) was incubated at 37° C on both host cell types. Because this virus is now identical to the original SPI-1 T309R mutant, it has lost the ability to form plaques on A549 cells.

Figure 18 shows a procedure for host range (hr) marker rescue. In Figure 18, other extragenic suppressors that were not temperature sensitive were mapped using A549 cells as a non-permissive host to select for recombinant viruses. Using an infection/transfection protocol and a suppressor DNA library, viruses containing the suppressor allele were amplified under liquid media, then assayed on fresh A549 cells.

Figure 19 shows a PCR library of 5kb products spanning the rabbitpox genome. As an alternative to generating multiple cosmid libraries, a more efficient PCR-based approach was developed in order to synthesize an effective library of 5kb products spanning the entire genome, using a series of primers with a common annealing temperature (Tm) as a reagent for each unique viral DNA template isolated. Such 5kb PCR products can be pooled together to simulate larger DNA fragments for coarse mapping, shown by the five longer lines. An agarose gel loaded with 5 µl of PCR product from 20 different primer sets is shown at the top of Figure 19, demonstrating the consistency and yield of 5kb PCRs generated in a single reaction.

Figure 20 shows an example of host range marker rescue on A549 cells. In this example, only the 5kb product #11 (viral suppressor DNA template) was able to rescue the host range of the RPV SPI-1 T309R (*hr*) mutant. This *hr* marker rescue was repeated with smaller PCR products from *hr* suppressor DNA, and only one 1.5kb fragment that overlapped the open reading frames D4R and D5R was able to restore host range to the *hr* mutant virus. Transfection with a wild type DNA fragment covering the same region serves as a negative control. DNA sequencing of the D4-D5 region of the viral suppressor genome revealed a mutation in the promoter region of D5R. One other suppressor mutant also mapped to D5R, but with a mutation in the coding region of the gene (A330T). D5R is an NTPase, essential in viral DNA replication, which is believed to participate in a multi-protein complex that includes the viral DNA polymerase (Evans, E. *et al.*, *J. Virol.*, 69(9):5353-5361).

Figure 21 shows how a standard viral plaque assay can demonstrate the host range phenotype of virus. (Left panel) Wild type rabbitpox virus can form plaques on A549 cells equally as well as on CV-1 cells, whereas the two examples of SPI-1 host range mutant do not. When rabbitpox virus containing the SPI-1 T309R host range mutation was used to infect A549 cells, spontaneous revertants arose containing second site mutations (in other genes) that suppress the original SPI-1 mutation. These revertants

were purified on A549 cells and were labeled Sup1, Sup2, and Sup3. (Right panel) The restored ability of these suppressor mutants to form plaques on A549 cells is shown in comparison with an equal dilution of each virus on CV-1 cells.

Figure 22A-22C shows properties of RPV Sup2 and Sup3 proteins, which are extragenic suppressors of RPV SPI 1 T309R. The Sup2 mutation is indicated as D5R A330T. Partial alignment surrounding the Sup2 locus in D5R gene homologs of other poxviruses from a variety of genera is shown (Figure 22C). Part of this region is predicted to form a helix secondary structure using a three-dimensional position specific scoring matrix (3D PSSM) (Evans, E., *et al., J. Virol.*, 69(9):5353-5361; Huntington, J.A., *et al., Nature*, 407:923-926), but its function is unknown. RPV = rabbitpox (orthopox), MYX = myxoma (leporipox), YLDV = Yaba like disease virus (yatapox), MCV = molluscum contagiosum (molluscipox), FPV = fowlpox (avipox). The Sup3 T to G mutation is upstream of the ATG start site for D5R (Figure 22A). * indicates proposed sites of transcription initiation that have been previously mapped by S1 nuclease protection assays (Lackner, C. A., and R. C. Condit, *J. Biol. Chem.*, 275:1485-1494; Phillips, J. E., *et al., J. Biol. Chem.*, 269:16696-16700).

Brief Description of Sequences

SEQ ID NOs. 1-98 are PCR primers of the subject invention (sequences are listed in Table 1), obtained from vaccinia virus and rabbitpox virus genomic sequences.

Detailed Disclosure

In one aspect, the subject invention provides an isolated polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID

NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or a variant of any of the foregoing.

In another aspect, the subject invention provides an isolated polynucleotide consisting of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID

NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or a variant of any of the foregoing.

In another aspect, the subject invention provides a set of polymerase chain reaction (PCR) primers useful for creating a DNA library of a microorganism, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism. In one embodiment, the primer pairs comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or variants of any of the foregoing.

In another aspect, the subject invention provides a method for designing a set of PCR primers for creating a DNA library of a microorganism, said method comprising providing a set of PCR primers, wherein the set of PCR primers comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism. In one embodiment, the primer pairs comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or variants of any of the foregoing.

In another aspect, the present invention provides a set of PCR primers useful for detecting the presence of the microorganism in a sample, wherein said set comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism. In one embodiment, the primer pairs comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or variants of any of the foregoing.

In another aspect, the present invention provides a method for designing a set of PCR primers for detecting the presence of the microorganism in a sample, said method comprising providing a set of PCR primers, wherein the set of PCR primers comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one

antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism. In one embodiment, the primer pair(s) comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or variants of any of the foregoing.

In another aspect, the present invention provides a method of producing a DNA library of a microorganism, wherein the method comprises: providing a sample containing the nucleotide sequence of the microorganism; annealing the set of PCR primers (such as those of the subject invention) to the nucleotide sequence of the microorganism; and amplifying the nucleotide sequence of the microorganism with the set of PCR primers (such as those of the subject invention) in separate polymerase chain reactions, wherein each polymerase chain reaction is carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon, thereby providing a DNA library comprising the resulting amplicons. In another aspect, the present invention provides a DNA library of a microorganism produced by the method of the present invention.

In another aspect, the present invention provides a method of mapping a mutation of a mutant microorganism, wherein the mutation confers growth under an otherwise non-permissive condition, said method comprising: transfecting a mutant amplicon into a host containing a microorganism lacking the mutation, wherein the mutant amplicon is from a DNA library of a mutant microorganism; recombining the mutant amplicon with the genome of the microorganism lacking the mutation, resulting in a recombinant microorganism; monitoring the growth of the recombinant microorganism; and sequencing the mutant amplicon if growth of the recombinant microorganism is observed.

In another aspect, the present invention provides a method of mapping a mutation of a mutant microorganism, wherein the mutation confers growth under an otherwise non-permissive condition, said method comprising: transfecting an amplicon into a host containing a mutant microorganism, wherein the amplicon is from a DNA library of a microorganism lacking the mutation; recombining the amplicon with the genome of the mutant microorganism, resulting in a recombinant microorganism; monitoring the growth of the recombinant microorganism; and sequencing the mutation of the mutant microorganism if growth of the recombinant microorganism is observed.

In another aspect, the present invention provides a method of mapping a mutation of a mutant microorganism, wherein the mutation inhibits growth under an otherwise permissive condition, said method comprising: transfecting a mutant amplicon into a host containing a microorganism lacking the mutation, wherein the mutant amplicon is from a DNA library of a mutant microorganism; recombining the mutant amplicon with the genome of the microorganism lacking the mutation, resulting in a recombinant microorganism; monitoring the growth of the recombinant microorganism; and sequencing the mutant amplicon if growth of the recombinant microorganism is not observed.

In another aspect, the present invention provides a method of mapping a mutation of a mutant microorganism, wherein the mutation inhibits growth under an otherwise permissive condition, said method comprising: transfecting an amplicon into a host containing a mutant microorganism, wherein the amplicon is from a DNA library of a microorganism lacking the mutation; recombining the amplicon with the genome of the mutant microorganism, resulting in a recombinant microorganism; monitoring the growth of the recombinant microorganism; and sequencing the mutation of the mutant microorganism if growth of the recombinant microorganism is not observed.

The mutation mapped according to the methods of the present invention can be, for example, host range, antibiotic resistance, drug resistance, drug dependency, or temperature resistance.

In another aspect, the present invention provides a method of creating an electrophoresis pattern of at least a portion of the genomic sequence of a microorganism for the purpose of detecting the presence of the microorganism in a sample, said method comprising: providing a sample containing the nucleotide sequence of the microorganism; annealing the set of PCR primers (such as those of the present invention) to the nucleotide sequence of the microorganism; amplifying the nucleotide sequence of the microorganism with the set of PCR primers (such as those of the present invention) in separate polymerase chain reactions, wherein each polymerase chain reaction is carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon; resolving the amplicons by electrophoresis; and obtaining an image of the amplicons resolved by electrophoresis, wherein the image is the electrophoresis pattern of at least a portion of the genomic sequence of a microorganism. In another aspect, the present

invention provides an electrophoresis pattern of the nucleotide sequence of a microorganism produced by the method of the subject invention.

In another aspect, the present invention provides a method for determining the presence of a microorganism in a sample, said method comprising: providing a sample for testing the presence of the genomic sequence of a known microorganism; annealing the set of PCR primers (such as those of the present invention) to any homologous polynucleotide sequence in the sample; amplifying the sequence of any homologous polynucleotide sequence in the sample with the set of PCR primers in separate polymerase chain reactions, wherein each polymerase chain reaction is carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon; resolving the amplicons by electrophoresis; obtaining an image of the amplicons resolved by electrophoresis, wherein the image is the electrophoresis pattern of any homologous polynucleotide sequence in the sample; and comparing the electrophoresis pattern of any homologous polynucleotide sequence in the sample with the electrophoresis pattern of at least a portion of the genomic sequence of a microorganism produced by the method according to the present invention.

In another aspect, the present invention provides a kit useful for creating a DNA library of a microorganism comprising: a set of PCR primers, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism; and at least one reagent selected from the group consisting of a buffer, a polymerase (*e.g.*, a thermostable DNA polymerase), deoxyribonucleotides, and an appropriate divalent cation. In one embodiment, the set of PCR primers is produced using the method of the present invention. In a specific embodiment, the set of PCR primers comprises nucleotide sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or a variant thereof.

In another aspect, the present invention provides a kit useful for creating a DNA library of a microorganism comprising: a set of PCR primers, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying

polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism; and at least one reagent selected from the group consisting of a buffer, a polymerase (*e.g.*, a thermostable DNA polymerase), deoxyribonucleotides, an appropriate divalent cation, and a positive control template. In one embodiment, the set of PCR primers is produced using the method of the present invention. In a specific embodiment, the set of PCR primers comprises nucleotide sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or a variant thereof.

In another aspect, the present invention provides a kit useful for detecting the presence of a microorganism in a sample comprising: a set of PCR primers, wherein said set comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism; at least one reagent selected from the group consisting of a buffer, a polymerase, deoxyribonucleotides, a magnesium salt, and a positive control template. In one embodiment, the set of PCR primers is produced using the method of the present invention. In a specific embodiment, the set of PCR primers comprises nucleotide sequences selected from the group consisting of SEQ ID NO:1 to SEQ ID NO:98, or a variant thereof.

DNA sequencing methods used to carry out the methods of the subject invention include, but are not limited to, the Sanger (dideoxy) method (Sanger, F., *Science*, 1981, 214(4526):1205-1210) and the Maxam-Gilbert method (Maxam A.M. and Gilbert W., *Methods Enzym.*, 1980, 65(1):499-560), and variations thereof.

Thus, according to the methods of the present invention, a genomic PCR library can be constructed by isolating genomic DNA (*e.g.*, viral DNA) from a biological or environmental sample, and generate overlapping PCR products that span the entire genome of the microorganism in a single reaction using only one optimized PCR condition for all primers. Specifically, all PCR reactions are run simultaneously using the same conditions of time and temperature, and using primers with similar characteristics of length, annealing temperature, and expected product size. Results of the PCR are visualized by loading the PCR products on a resolving gel. PCR products can be purified

in spin columns. Preferably, the PCR product size is approximately 5kb in length (*e.g.*, within the range of about 4.5 kb to 5.5 kb in size).

The methods of the present invention will now be described with respect to orthopoxvirus, but it should be understood that the methods of the present invention are equally applicable to other microbial agents, such as other viruses, bacteria, yeast, and transposons. The diagnostic methods of the present invention involve using primers developed for a PCR library to generate genomic libraries for other species of microorganism, such as other species of orthopoxvirus (*e.g.*, variola major-smallpox, various strains of vaccinia virus, cowpox, mousepox, monkeypox, variola minor). In carrying out the method, it is likely that many PCR products may not be generated. For example, in the case of orthopox viruses, this will be due to differences in the DNA sequence of the orthopoxvirus being tested from the sequence of the PCR primers (*e.g.*, oligonucleotides based upon vaccinia virus and rabbitpox virus sequence). An observable pattern (*e.g.*, an electrophoresis pattern or spectra pattern) may develop where some of the PCR products are made and some are not, depending upon which particular virus or viruses is being screened for. The pattern of PCR products observed can then be compared to known patterns of PCR products associated with particular orthopoxvirus.

A second aspect of this method relies upon a given PCR product being generated that is different in size than would be expected from vaccinia virus. For example, if PCR product #23 is 5kb in size for vaccinia virus, but 4kb for smallpox, the difference in size can be readily determined by standard DNA techniques. Therefore, as long as a product is generated, the size of the product alone may determine the presence of smallpox in a biological or environmental sample. The diagnostic methods of the present invention are particularly useful for distinguishing among closely related taxa of microorganisms, such as orthopoxvirus.

In one embodiment, the diagnostic method of the present invention involves isolating DNA (including target DNA) from a sample, such as blood. The isolated DNA can be suspended in solution (in H_2O, for example). A primer pair of the present invention is added to each vessel (*e.g.*, PCR tube) containing the DNA solution, wherein all of the primers have a similar predicted annealing temperature (Tm) (for example, within the range of about 55 °C to about 63 °C). The Tm of each primer within a pair should not differ from each other by more than about 2°C. The primers to be used are

selected based upon the microorganism being screened for. Examples of preferred primer pairs that can be utilized to distinguish from among orthopoxviruses are described in Example 2. Other PCR reagents, such as deoxynucleotides (dNTPs), enzymes, and buffer, will also be added. PCR amplification is then carried out, resulting in PCR products (amplicons), which are then resolved by electrophoresis in gels. The pattern of the resolved PCR products is analyzed to determine the presence of a characteristic PCR product or products, indicating the presence of the particular microorganism (*e.g.*, virus) of interest within sample. Advantageously, the analysis of the PCR product patterns can be conducted visually. However, computer-assisted image analysis of gels can also be conducted, using densitometry, for example, and automated scoring. In addition to the presence or absence of a particular PCR product, the sizes of the PCR products (as determined by gel migration) can be compared to a control, such as the PCR product pattern from a sample containing a known amount of the microorganism of interest. Optionally, the sequences of the resolved PCR products can be analyzed using restriction fragment length polymorphism (RFLP) methods, for example.

The nucleotide sequence of the PCR primers of the present invention is typically about 23 to about 28 bases in length. In one embodiment, the nucleotide sequence of the PCR primers of the present invention is about 26 bases in length. Examples of PCR primers and primer pairs of the present invention are listed in Tables 1 – 3 (*e.g.*, SEQ ID NOs. 1-98), and fragments and variants of those nucleotide sequences. The primers of the present invention will successfully function using a common annealing temperature during PCR (such as 53°C), because they were chosen by allowing an optimum annealing temperature within the range of about 55 °C to about 63 °C. Preferably, the common annealing temperature for each primer is about 57 °C. The primers of the present invention produce PCR products at least about 4.5 kb in size, which are yet another aspect of the present invention. Preferably, the primers of the present invention produce PCR products in the range of about 4.5 kb to about 5.5 kb in size.

Table 1

5kb PCR Primers for Rabbitpox/Vaccinia WR	
Primer pair #1 5.1 kb (ITR) 35K to CPV CHO Tm=57 IDT 3 gac aca cgc ttt gag ttt tgt tga at (SEQ ID NO. 1) IDT 4 aaa tca gaa tgt tac agc acc ggt ta (SEQ ID NO. 2)	#22 4.9kb: H4-D1 Tm=61-59 FS 331 ttt ttg gaa atg gga ata ccg atg tg (SEQ ID NO. 43) FS 332 aat aat gga tag caa act gcc agt cg (SEQ ID NO. 44
#2 4.9kb CPV CHO to SPI-1 Tm=56 IDT 5 tag tcg tgc aga gga tag ttg ttg tc (SEQ ID NO. 3) IDT 6 cta aat cat gga gca gct ggt tct ac (SEQ ID NO. 4)	#23 5.0kb: D1-D5 (Guanyl tra.) Tm=60-57 FS 333 cga gga taa act atc gga tgt ggg ac (SEQ ID NO. 45 FS 334 ttg gaa agt agt caa cgg aag agt ga (SEQ ID NO. 46
#3 5.4kb SPI-1 to CPV 77K HR Tm=57 FS 396 gac ctt cgt ccg ata tac cat cga aa (SEQ ID NO. 5) IDT 9 cta gaa tcc ggt tca gat gtc aac at (SEQ ID NO. 6)	#24 5.0kb: D5-D10 Tm=57-55 FS 335 gac aga acc ttg tgt cat tgg aag ac (SEQ ID NO. 47) FS 336 tca gca gta gct gga tct aga gaa aa (SEQ ID NO. 48
#4 5.3kb CPV 77K HR to C7L Tm=55 IDT 10 tca cgt aat tct gga tta ttg gca (SEQ ID NO. 7) IDT 11 aca gca cga att cga cat cat tat t (SEQ ID NO. 8)	#25 5kb: D9R-D13L (rifamp.r) Tm=60-57 FS 337 gca tgg cgt gtt tta tta atc aat cg (SEQ ID NO. 49) FS 338 aaa ctc cgt ttg atg tgg aag ata ca (SEQ ID NO. 50)
#5 5.0kb C7L to C2L Tm=55 IDT 12 gtg ttt gaa gag ctc gtt ctc atc a (SEQ ID NO. 9) IDT 13 gcc ata ata att gat gtt ccg cc (SEQ ID NO. 10)	#26 4.9kb: D13L-A4L(mem. core) Tm=57 FS 339 tct ttt tca tct tgt gag tac cct gg (SEQ ID NO. 51) FS 340 tat cca acc gct aca acc aac tat tc (SEQ ID NO. 52)
#6 4.4kb C2L-M1L Tm=57 IDT 54 gtt ttt cac agt tgc tca aaa acg at (SEQ ID NO. 11) IDT 55 atc caa acg cgt gtg ata aac ata at (SEQ ID NO. 12)	#27 5.0kb: A4L-A8R(33.6K) Tm=57 FS 341 agg tgt agg agt aca tac agt ggc ca (SEQ ID NO. 53 FS 342 tgt tca tct cga ata gcc aga gct ac (SEQ ID NO. 54)
#7 5.0kb M1L-K3L Tm=57 IDT 52 ttc gct cat aca tgt atg acc gtt aa (SEQ ID NO. 13) IDT 53 cct att att tat tcg aag gcg ggt aa (SEQ ID NO. 14)	#28 4.9kb: A8R-A12L(virion) Tm=57 FS 343 taa ttt gga ggc ctc cgt aga act ag (SEQ ID NO. 55) FS 344 tat cga ctc agt taa tgc tgg gaa aa (SEQ ID NO. 56)
#8 5.1kb K3L-F3L Tm=57 IDT 50 ctg tat ttt gaa ttc caa tca cgc at (SEQ ID NO. 15) IDT 51 tcg cac aca ttt acg tca aaa ata ca (SEQ ID NO. 16)	#29 4.9kb: A11R-A18R Tm=57-56 FS 345 att gtc ata aac att atc gag cag gc (SEQ ID NO. 57) FS 346 tag ttc tgt ttc tcg aca tac cct cc (SEQ ID NO. 58)
#9 5.1kb F3L-F10L Tm=56 IDT 48 ctg gaa cat tta gtt cat cgc ttt ct (SEQ ID NO. 17) IDT 49gcc aca aaa tta tat agt ccc atg ga (SEQ ID NO. 18)	#30 5kb: A18R-A24R(rpo132) Tm=56=57 FS 347 gtt ttt ttg agc cat att cca cag ac (SEQ ID NO. 59) FS 348 gat aga caa ctt ctg gag gca tat cg (SEQ ID NO. 60)
#10 5.0kb F10L-F13L Tm=58 IDT 46 aag tca aag tcg ttt aat gcc gat tt (SEQ ID NO. 19) IDT 47 ggc gcg ctt att ttt gat aaa cta aa (SEQ ID NO. 20)	#31 5kb: A24R-A26L (spansATI) Tm=57 FS 376 ctc cag att act cac cta taa tcg cg (SEQ ID NO. 61) FS 377 gaa ctt gaa ctc agt cgt atg tgg ct (SEQ ID NO. 62)
#11 5.0kb F13L-E2L Tm=59 IDT 44 cgc aga gca taa att caa cca tga at (SEQ ID NO. 21) IDT 45 ttc tgt cca atg atg atg aaa cgg tt (SEQ ID NO. 22)	#32 5.4kb: A26L-A29L Tm=59 FS 378 ttt cgg cat tgg ttt cat tat tac gt (SEQ ID NO. 63) FS 379 taa aat ggc cct tgt taa aca ttg ga (SEQ ID NO. 64)
#12 5.1kb E2L-E6R Tm=58 IDT 42 tca tgt tca cta ctg gtg tcc acg at (SEQ ID NO. 23) IDT 43 ccg caa act cta tgc ctg tat ctt tc (SEQ ID NO. 24)	#33 4.5kb: A29L-A36Ra.tail Tm=58-57 FS 380 caa aca cga ttt aaa atc aaa cca cg (SEQ ID NO. 65) FS 381 aat gaa cgg att tga ctt gct aca aa (SEQ ID NO. 66)
#13 5.0kb E6-E9 Tm=57 FS 317 tac gat gtt gta aag tgt acg aag cg (SEQ ID NO. 25) FS 318 agt tag aga aat gac gtt cat cgg tg (SEQ ID NO. 26)	#34 5.0kb: A36R-A41L(early glyc) Tm=58 FS 349 atg atg ctg gta cct ctt atc acg gt (SEQ ID NO. 67) FS 382 cta gac gaa ccc ctc aga caa aca ac (SEQ ID NO. 68

5kb PCR Primers for Rabbitpox/Vaccinia WR	
#14 4.9kb E9 (DNA Pol)-I1 Tm=59 FS 219 ttt gtt ttg gag caa ata cct tac cg (SEQ ID NO. 27) FS 220 cga gag tgg ttg aat gtt tga ctg tg (SEQ ID NO. 28)	#35 5.0kb: A41L-A47L Tm=58 FS 383 att tta cgt ggt tga cca ttc ttg gt (SEQ ID NO. 69) FS 384 gca acc atc caa aga ttt tca tct ct (SEQ ID NO. 70)
#15 4.9kb O2- RNA reductase Tm=60-61 FS 319 aaa tag tca cgc aat tca ttt tcg gg (SEQ ID NO. 29) FS 320 tgc ttt tga tgg taa ttt ctg gtg cc (SEQ ID NO. 30)	#36 5.2kb: A47L-A52R Tm=58 FS 385 atg ttt gac ttt atg gtt aga ccc gc (SEQ ID NO. 71) FS 386 tct cat ttc tcc aaa cat cta cca cg (SEQ ID NO. 72)
#16 4.9kb: RNA red.–I8 RNA helic. Tm=57 FS 321 ggc ata atc cgg atg ttg tgt agt ac (SEQ ID NO. 31) FS 322 gta gcg ttt gtt ccg tta tcg aca cc (SEQ ID NO. 32)	#37 5.9kb: A52R-B1R Tm=57 FS 387 acg gtg act act agg agg gaa aat ga (SEQ ID NO. 73) FS 388 ctc tag gtg cat att gca aac tgg tc (SEQ ID NO. 74)
#17 4.9kb: I8R-G5.5 Tm=57 FS 323 atc taa cac tcc ccg aag att tgt tt (SEQ ID NO. 33) FS 324 tat cgt tcg tga gaa ata tct ttg cc (SEQ ID NO. 34)	#38 5.7kb: B1R-B6R Tm=57 FS 389 tag att agg tgc aga tct aga tgc gg (SEQ ID NO. 75) FS 390 cgt gcc aat agt agt tag aga tgc gt (SEQ ID NO. 76)
#18 5.1kb: G5-L3 Tm=56 FS 325 act cgt ata ttc ctc ctt gtc aat gc (SEQ ID NO. 35) FS 326 tta tgg cag gtg aga tgt ttg tta ga (SEQ ID NO. 36)	#39 5.0kb: B6R- B13R (Spi-2) Tm=57 FS 391 gga tgt tga tat cta cga tgc cgt ta (SEQ ID NO. 77) FS 392 aca tgc cta atc aca tag atg aac gg (SEQ ID NO. 78)
#19 5.0kb: L1-J4 Tm=63-60 FS 221 cat taa ggc gtt gat gca att gac ga (SEQ ID NO. 37) FS 222 tgg ttc tcc ata atc atc atc aac cg (SEQ ID NO. 38)	#40 5.0kb: B13R (Spi-2) -B17R Tm=53 FS 393 aac ttt tca atc ata gaa ctg cca ta (SEQ ID NO. 79) FS 394 ata aag atc gtt aat tgt cag cat gt (SEQ ID NO. 80)
#20 5kb: J3-J6 Tm=56 FS 327 ttc aca tgt act tta tgc tga gga cc (SEQ ID NO. 39) FS 328 cat gag aag acc caa gtc gat aaa gt (SEQ ID NO. 40)	#41 4.0kb B17R-B19R (ank.like) Tm=57 FS 395 gaa tgt gat aag atc gtt ggt gga tc (SEQ ID NO. 81) IDT 7 aca tca tat act cga gga cgg cat ta (SEQ ID NO. 82)
#21 5.0kb: J6-H4 Tm=59 FS 329 tca ttg taa agg aat ggg gta tgg aa (SEQ ID NO. 41) FS 330 aga aca gaa tat tga cgc gga tga tc (SEQ ID NO. 42)	#42 4.5kb B19R-CPV CHO HR Tm=56 IDT 8 ggt tac ttt gaa gga cgt aca cca ct (SEQ ID NO. 83) IDT 5 tag tcg tgc aga gga tag ttg ttg tc (SEQ ID NO. 3)

Table 2

For VVwr left and right ends: V1-V7 replaces #1-9; V41-V42 replaces #41-42	**V5 4.9kb:** N2L-K2L (SPI-3) Tm=57 IDT 67 aca ttt gaa ctc atc gta cag gac gt (SEQ ID NO. 91) IDT 68 tac cta tcg tct gca agg att tac ca (SEQ ID NO. 92)
V1 5.1kb: 35K – B25/C10 Tm=58-57 IDT 3 gac aca cgc ttt gag ttt tgt tga at (SEQ ID NO. 1) IDT 60 aac ttc cga tgg aag aca att ctg at (SEQ ID NO. 84)	**V6 5.4kb:** K2L-F3L Tm=58 IDT 69 ttg cgt gtt tta gtg ata tca aac gg (SEQ ID NO. 93) IDT 70 ggt gga tgg atg aac aat gaa ata ca (SEQ ID NO. 94)
V2 5.3kb: B25/C10-C9L Tm=57 IDT 61 ggc aat tgt ttt acg tcc agt taa ca (SEQ ID NO. 85) IDT 62 atg gtt aat ags tac tgg cgc aga ca (SEQ ID NO. 86)	**V7 5.5kb:** F3L-F10L Tm=57 IDT 71 atc aat tct gga tta tcc ctc gga ta (SEQ ID NO. 95) IDT 72 aat tac tat ccc act ttt atc cgg ca (SEQ ID NO. 96)
V3 5.3kb: C9L-C4L Tm=59-58 IDT 63 gaa cgc gta cga gaa aat caa atg tc (SEQ ID NO. 87) IDT 64 tca aat gta acg gta tcg tct acc ga (SEQ ID NO. 88)	**V41 5kb:** B18R-B22R (Spi-1) Tm=57 IDT 395 gaa tgt gat aag atc gtt ggt gga tc (SEQ ID NO. 97) IDT 396 gac ctt cgt acg ata tac cat cga aa (SEQ ID NO. 98)
V4 5.0kb: C4L-N2L Tm=58 IDT 65 ccg tat ctc caa caa gca cgt agt aa (SEQ ID NO. 89) IDT 66 ttt acc cga agg tag tag cat gga tc (SEQ ID NO. 90)	**V42 3.7kb:** B22R-B25/C10 Tm=57 IDT 6 cta aat cat gga gca gct ggt tct ac (SEQ ID NO. 4) IDT 61 ggc aat tgt ttt acg tcc agt taa ca (SEQ ID NO. 85)

The use of methods of the present invention to generate fragments of genomic DNA for use in genetic mapping by marker rescue has already proven to have profound advantages over the more conventional use of cosmid libraries in vaccinia or rabbitpox virus. With the availability of genomic sequences for several large DNA viruses, design of oligonucleotide primers for amplification of approximately 5kb or larger PCR products can be easily adapted for use in other species, in lieu of other forms of vector-based genomic libraries. Genomic sequence data available online provides use of these PCR primers in other closely related species of orthopoxviruses with very little modification. Creating genomic libraries by PCR for viruses other than DNA viruses, or any other cellular genomic fragment on the order of 200kb in size (bacterial or eukaryotic) that would be of consistent interest in mapping mutations, is a reasonable and predicted extension of this concept. The selectable condition for the mutations mapped in the system is growth of RPV in A549 cells, which will continue to contribute to our understanding of mechanisms involved in poxviral host range. Perhaps more importantly, however, is the extension of the PCR-based genomic library approach of the present invention to mapping mutations responsible for any other selectable growth condition, such as the adaptation of a virus for replication in a foreign host or resistance to antiviral drugs.

Among the techniques useful in the nucleic acid-based methods of the invention are enzymatic gene amplification (or polymerase chain reaction (PCR)), Southern blots, Northern blots, or other techniques utilizing nucleic acid hybridization for the identification of polynucleotide sequences in a sample, which are familiar to those of ordinary skill in the art. Nucleic acids and/or vectors can be introduced into host cells by well-known methods, such as, calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection (see, for example, Sambrook *et al.* [1989] *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

In another aspect, the present invention concerns kits useful for creating DNA libraries and diagnostic kits for determining the identity of one or more unknown microorganisms present in a sample, such as a biological or environmental sample. The diagnostic kits of the present invention include one or more primer pairs of the present invention, which are useful for detecting a target nucleic acid within a sample. The kits useful for creating DNA libraries of the present invention include two or more primer pairs. The primer pairs in each kit have a similar predicted annealing temperature (TM) (within about 2 °C), which can be within the range of about 55 °C to about 63 °C. Preferably, the common annealing temperature is about 57 °C. The primers in the kits of the present invention produce PCR products at least about 4.5 kb in size. Preferably, the primers produce PCR products in the range of about 4.5 kb to about 5.5 kb in size.

In one embodiment, the kit contains 4 to 7 PCR primer pairs. Each diagnostic kit can be designed for the identification of one or more microorganisms of interest. For example, in the case of a kit for the detection of variola virus, which causes smallpox, the kit can contain one or more primer pairs selected from the group consisting of V3, V6, 37, and V41, as described in Tables 1-3 and Example 2. In the case of a kit for the detection of monkeypox virus, the kit can contain one or more primer pairs selected from the group consisting of 31, 37, and 40.

Diagnostic kits of the present invention can further include one or more primer pairs useful as positive or negative controls. For example, primer pairs V1 and V42 can be included as negative controls. Primer pairs 14 and 28 can be included as positive controls. Written and/or graphically represented PCR product patterns to be expected

with positive results (indicating presence of the microorganism(s) in the sample) can also be included within each kit. The diagnostic kits of the present invention can include compartments for containing each primer pair, and other components of the kits, such as various PCR reagents. Compartments can be composed of any solid material, such as metal or plastic, and can include containers or vessels for holding reagents. The kits can further include various packaging for the compartments containing the various components of each kit.

The specificity of any given PCR reaction relies heavily, but not exclusively, on the identity of the primer sets. The primer sets are pairs of forward and reverse oligonucleotide primers that anneal to a target DNA sequence to permit amplification of the target sequence, thereby producing a target sequence-specific amplicon. As used herein, the terms "derivative" or "variant" of a specified oligonucleotide are used interchangeably to refer to an oligonucleotide that binds to the same target sequence as the specified oligonucleotide and amplifies the same target sequence to produce essentially the same amplicon as the specified oligonucleotide but for differences between the specified oligonucleotide and the variant. The variant can differ from the specified oligonucleotide by insertion, deletion, and/or substitution of any residue of the specified sequence so long as the variant substantially retains the characteristics of the specified sequence in its use for the same purpose as the specified sequence.

A variant nucleotide sequence, for the purposes of the present invention, such as a variant of those sequences in Tables 1-3, encompasses a nucleotide sequence having a percentage identity with the bases of the nucleotide sequences of between at least (or at least about) 50.00% to 99.99% (inclusive). The aforementioned range of percent identity is to be taken as including, and providing written description and support for, any fractional percentage, in intervals of 0.01%, between 50.00% and 99.99%. These percentages are purely statistical and differences between two nucleic acid sequences can be distributed randomly and over the entire sequence length.

In various embodiments, variant sequences exhibiting a percentage identity with the bases of the nucleotide sequences of the present invention (*e.g.*, those nucleotide sequences in Tables 1 –3) can have 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87,

88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identity with the polynucleotide sequences of the instant invention.

Nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman [1988] *Proc. Natl. Acad. Sci. USA* 85(8):2444-2448; Altschul *et al.* [1990] *J. Mol. Biol.* 215(3):403-410; Thompson *et al.* [1994] *Nucleic Acids Res.* 22(2):4673-4680; Higgins *et al.* [1996] *Methods Enzymol.* 266:383-402; Altschul *et al.* [1990] *J. Mol. Biol.* 215(3):403-410; Altschul *et al.* [1993] *Nature Genetics* 3:266-272).

The subject invention also provides nucleotide sequences complementary to any of the polynucleotide sequences disclosed herein. Thus, the invention is understood to include any DNA whose nucleotides are complementary to those of the sequence of the invention, and whose orientation is reversed (*e.g.*, an antisense sequence).

The present invention further provides fragments of the polynucleotide sequences provided herein. Representative fragments of the polynucleotide sequences according to the invention will be understood to mean any nucleotide fragment having at least 11 or 12 successive nucleotides, preferably at least 15 successive nucleotides, and still more preferably at least 18 or at least 23 successive nucleotides of the sequence from which it is derived. The upper limit for such fragments is the total number of polynucleotides found in the full-length sequence. It is understood that such fragments refer only to portions of the disclosed polynucleotide sequences that are not listed in a publicly available database or prior art references.

As used herein, the term "polymerase chain reaction" refers to the process of amplifying nucleic acids *in vitro*, as disclosed in U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188; and Saiki *et al.*, *Science*, 230:1350-1354 (1985). DNA (deoxyribonucleic acid) may be amplified by thermally cycling a liquid reaction mixture according to a PCR protocol. Typically, each cycle of amplification comprises three steps: (a) a denaturing step; (b) an annealing step; and (c) an extension step. A typical PCR reaction mixture comprises nucleic acid template, two amplification primers complementary to the two 3' borders of the duplex segment (target) to be amplified, four deoxyribonucleotides (dATP, dCTP, dTTP, and dGTP), enzymes, and an appropriate

buffer. The duplex DNA molecule is denatured into two complementary single strands. The primers then anneal to the strands and nucleoside monophosphate residues are then linked to the primers in the presence of an enzyme such as a thermostable DNA polymerase to create a primer extension product. After primer extension, twice as much duplex DNA molecules exist. This process is repeated, each time approximately doubling the amount of DNA present. The result is an exponential increase in the concentration of target DNA, known as "amplification" of the target DNA.

The PCR carried out in the methods of the present invention can be carried out as disclosed herein and performed in reaction vessels (*e.g.*, composed of glass, plastic, metal), such as plastic microcentrifuge tubes or test tubes which are placed in an instrument containing a thermally controlled heat exchanger. Examples of these instruments are disclosed in U.S. Patent No. 5,038,852, which is hereby incorporated by reference in its entirety. The heat exchanger is typically a metal block; however, hot air ovens and water baths can also be used. The temperature of the reaction mixture in the reaction tubes is changed in a cyclical fashion to cause denaturation, annealing and extension reactions to occur in the mixture. Typically, a PCR reaction is carried out with one primer pair per reaction vessel (*e.g.*, tube) and results in an amplicon.

As used herein, the term "primer" refers to an oligonucleotide that hybridizes with a nucleic acid template and provides a free 3' hydroxyl group that is used by DNA polymerase to initiate extension of DNA fragments complementary to the template. In PCR, for example, each primer is used in combination with another primer forming a "primer pair"; this pair flanks the targeted sequence to be amplified. A primer "set" refers to one or more primer pairs. In some embodiments, a primer set is two or more primer pairs. Examples of primers of the subject invention are listed in Tables 1-3.

The term "genomic DNA" refers to chromosomal DNA and, in the case of eukaryotic DNA, can include introns. An intron is an intervening non-coding sequence of DNA within a gene that is transcribed into hnRNA, but is then removed by RNA splicing in the nucleus, leaving a mature mRNA, which is then translated in the cytoplasm of the cell. The regions at the ends of an intron are self-complementary, allowing a hairpin structure to form naturally in the hnRNA. In the case of poxvirus, genomic DNA exists entirely in the cytoplasm (outside of the nucleus) of infected cells, is transcribed into mRNA by virus-encoded enzymes, and the resultant mRNA is not spliced. The genomic

DNA can be a portion (fragment) of the microbial agent's genetic material, or the entire amount.

As used herein, the term "vector" refers to a DNA molecule, such as a plasmid, cosmid, phagemid, or bacteriophage or other viral entity, which typically has a capability of replicating in a host cell and which is used to transform cells for genetic modification. Vectors typically contain one or more restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Appropriate marker genes typically include genes that provide various antibiotic resistance. A variety of markers are available to those of ordinary skill in the art.

As used herein, the term "reagent", or grammatical variations thereof, within the context of enzymatic reaction mixtures, such as a reverse transcription and PCR reaction mixture, includes any compound or composition that is added to the reaction mixture including, without limitation, enzyme(s), nucleotides or analogs thereof, primers and primer sets, buffer, salts, and co-factors. As used herein, unless expressed otherwise, the term "reaction mixture" includes all necessary compounds and/or compositions necessary to perform that enzymatic reaction, even if those compounds or compositions are not expressly indicated.

As used herein, the term "sample" refers to a medium (solid, liquid, gas, *etc.*) potentially containing target genetic material (*e.g.*, DNA) of the microbial agent of interest. Suitable samples include biological samples, such as blood, hair, saliva, sweat, and tears, and environmental samples, such as air, soil, and water. The sample may itself be genetic material (*e.g.*, DNA) in an isolated state.

As used herein, the term "microorganism" in the context of the subject invention refers to microbial agents bearing genetic material, including prokaryotic and eukaryotic microorganisms, such as bacteria and yeast; as well as replicons, such as viruses and transposons.

As used herein, the term "genetically related population" refers to any grouping of microorganisms possessing multiple or single genotypic or phenotypic characteristics of sufficient similarity to allow the organisms to be classified as a single genus, species, or

subspecies. An example of a genetically related population includes the group of viruses classified as orthopoxviruses.

The terms "comprising", "consisting of", and "consisting essentially of" are defined according to their standard meaning and may be substituted for one another throughout the instant application in order to attach the specific meaning associated with each term.

It should be understood that various optimization techniques can be utilized to enhance the method of the subject invention, such as increasing the yield of DNA products obtained from the PCR by modifying the temperature cycling conditions, or by substituting a different reagent, such as a more flexible, thermostable DNA polymerase (see, for example, Sambrook *et al.* [1989] *Molecular Cloning, A Laboratory Manual, Second Edition*, Cold Spring Harbor Press, N.Y.; and Ausubel *et al.* [1989] *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y., each incorporated herein by reference in its entirety).

All patents, patent applications, provisional applications, and publications referred to or cited herein, whether supra or infra, are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Materials and Methods

Cells and viruses. A549, PK15, and CV-1 cells were obtained from the American Type Culture Collection. S1a cells (HeLa-S1a) were obtained from Dr. Toni Antalis (Queensland Institute of Medical Research) (Bayliss, C. D. and Condit, R. C., *Virology*, 194:254-262). Monolayers of CV-1 African green monkey kidney cells, PK15 pig kidney cells, and A549 human lung cells were grown in minimum essential media (MEM) with Earl's salts and 2.2 g of $NaHCO_3$ per liter. S1a cells were maintained in RPMI Medium 1640 with L-glutamine (GIBCO), plus 2.0 g of $NaHCO_3$ per liter, and 0.3 g of G418 sulfate per liter (Geneticin, GIBCO). All media was supplemented with 2 mM L-glutamine, 50 U of penicillin G per ml, 50 mg of streptomycin per ml, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids (GIBCO), and 5% or 10% fetal bovine serum (FBS). All viruses used are derivatives of RPV (rabbitpox virus, Utrecht strain).

RPV mutants in the SPI-1 gene (ΔSPI-1, T309R, and F322A) which cannot form plaques on A549 cells have been described (Brum, L. M., *et al.*, *Virology*, 315:322-334) and were provided by Dr. Kristin Moon. SPI-1 F322A is a mutation at the P1 residue of the reactive site loop (RSL), which normally determines target protease recognition and specificity (Biggs, J. R., *et al.*, *Proc. Natl. Acad. Sci. USA*, 98:3814-3819), and removes SPI-1 activity against cathepsin G *in vitro*.

Plaque assays. Confluent monolayers of cells in either 6-well or 12-well plates were infected with serial dilutions of sonicated virus in MEM, and incubated at 37°C. 2 hr post infection the media was replaced with MEM + 5% FBS in 0.5% agarose at 43°C, and incubation continued at 37°C (or 41°C to assay for temperature sensitivity). 4 to 6 days post infection, the agarose overlay was removed and the cells were stained with 2% crystal violet in 2% ethanol and 0.08% ammonium oxalate.

Selection for viral suppressor mutants by restored host range. Spontaneous phenotypic revertants of RPV SPI-1 T309R observed on A549 cells were picked and amplified in A549 cells in liquid growth medium. Revertant isolates that remained viable after three serial passages were cloned by serial plaque purification three times on A549 cells under agarose medium then amplified on CV-1 cells under liquid medium in 6-well plates. Restoration of host range for each revertant virus clone was verified by plaque assay on A549 and CV-1 cells. DNA was isolated from each revertant with the DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol, and the SPI-1 gene was amplified by PCR and sequenced. Revertant viruses that retained the parental SPI-1 T309R allele were presumed to have an extragenic suppressor mutation.

Growth and spread of RPV. 6-well dishes of A549 cells were infected at a multiplicity of 1×10^{-5} PFU per cell (~30 PFU per well) with each mutant virus and incubated at 37°C. 2 hr post infection the cells were washed once with MEM then incubated in 3 ml MEM + 10% FBS. Samples were harvested at 12 hour intervals for 72 hr, and titered for RPV on CV-1 cells by plaque assay.

Gel mobility shift assay for protease-serpin interaction. Wild type or mutant SPI-1 DNA (T309R or T309C) was cloned into the pTM1 expression vector (Bayliss, C. D. and Condit, R. C., *J. Biol. Chem.*, 270:1550-1556), which contains a T7 promoter and an internal ribosome entry site, and verified by DNA sequencing. 400 ng of pTMI SPI-1 DNA from each clone provided the template for both synthesis and labeling of SPI-1

protein with 8 μCi ^{35}S-Met and 16 μl TNT-Master Mix (TNT-T7 Quick Coupled In Vitro Transcription and Translation System, Promega), which contains purified T7 RNA polymerase and rabbit reticulocyte lysate. Each 20 μl reaction was then incubated at 30°C for 90 min. 2 μl of each SPI-1 TNT product was incubated at 37°C for 90 min with purified human cathepsin G (Athens Biotech) in the range of 0 to 3×10^{-4} units per sample in a buffer of 100 mM Tris pH 8 / 10 mM $CaCl_2$. Protease inhibitory function is measured by the ability of ^{35}S-labeled SPI-1 protein to form highly stable high molecular weight complexes, observed by gel mobility shift in 10% SDS-polyacrylamide gel electrophoresis (Colamonici, O. R., *et al.*, *J. Biol. Chem.*, 270:15974-15978). Gels were fixed in 25% methanol, 7% acetic acid at 4°C for 30 min, amplified in "Amplify" solution (Amersham) at room temperature for 30 min, transferred to Whatman 3MM filter paper (Fisher Scientific) by vacuum at ~80°C for 2 hr with a gel dryer (Bio-Rad). Labeled SPI-1 protein in each gel was then detected by exposure to X-ray film for 4 to 48 hr at -80°C (X-Omat Blue, Kodak).

Marker rescue of temperature sensitive mutant (*sup-2*). Wild type virus DNA from either VV-WR cosmids or RPV PCR products was quantitated by fluorometry. 1 μg DNA was mixed with 6 μl PLUS Reagent (Invitrogen) in 125 μl MEM and incubated at room temperature (RT) for 15 min. The DNA was then mixed with 4 μl Lipofectamine (Invitrogen) diluted in 125μl MEM and incubated at RT for 15 min. Confluent monolayers of CV-1 cells in 6-well plates (3×10^6 cells per well) were transfected with each DNA-lipid mixture and simultaneously infected at 37°C with RPV *sup*-2 at a multiplicity of 0.003 PFU/cell in a final volume of 0.75 ml MEM. After 3 hr incubation the media was replaced with 0.5% agarose in MEM with 5% FBS and incubated at 41°C. 4 days post infection the agarose overlay was removed and the cells were stained with crystal violet.

Marker rescue of non-temperature sensitive mutants. 2 μg PCR product (*sup* or wild type) was mixed with 5 μl Lipofectamine2000 (Invitrogen) in 250 μl Opti-MEM (Invitrogen) and incubated at RT for 20 min. Confluent monolayers of A549 cells in 6-well plates were transfected with each DNA-lipid mixture and simultaneously infected at 37°C with 0.01 PFU/cell of RPV SPI-1 F322A, 0.01 PFU/cell of RPVΔSPI-1, or 0.0003 PFU/cell of RPV SPI-1 T309R (because of leakiness) in a final volume of 0.75 ml Opti-MEM. After 5 hr incubation, an additional 3 ml MEM + 10% FBS was added and

incubation continued at 37°C for 48 hr (SPI-1 F322A or ΔSPI-1) or 120 hr (SPI-1 T309R). The cells were harvested by scraping, disrupted by three freeze/thaw cycles, and stored at -80°C. Recombinant viruses were then titered by host range phenotype reversion in plaque assays on A549 cells.

Site-directed mutagenesis of RPV DNA polymerase (E9L). A 1 kb fragment of wild type RPV E9L DNA flanking the His142 codon was synthesized by PCR to generate restriction endonuclease sites for cloning into the pAlterEx1 vector (pAltE9), which is required for mutagenesis. E ORF E (RPV homolog of VACVgp085, GenBank Accession # NP_063713) is imbedded within E9L in the opposite orientation. The mutagenic oligonucleotide for disruption of E ORF E was FS359 (5'phosphorylated-gga cga gca ata ttt aac gaa gat taa caa tgg a-3'), which ultimately leads to a Cys to Arg mutation at codon 9 followed by a stop codon. A unique *Mbo*II restriction site is also produced. Mutagenesis of pAltE9 DNA with the FS359 oligonucleotide to effectively eliminate E ORF E was performed using the Altered Sites system (Promega) and cloned into RPV by *ts* marker rescue of RPV *sup*-2 (SPI-1 T309R, E9ΔH142) on CV-1 cells at 41°C. This yielded RPV SPI-1 T309R / ΔE ORF E, which was then plaque-purified and verified by DNA sequencing.

DNA synthesis assay. Infected cell lysates were prepared as previously described (Bird, P. I., *Results Probl. Cell. Differ.*, 24:64-89). Monolayers of CV-1 cells in 60 mm dishes (8 x 10^6 cells) were infected with 1.5 ml of each virus diluted in growth medium (without serum) for a multiplicity of 10 PFU per cell. After 90 min incubation at 37°C, the cells were washed once with excess PBS (phosphate buffered saline: 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 4.3 mM Na_2HPO_4, pH 7.2), the media was replaced with 2 ml MEM + 10% FBS per well and the cells were incubated at either 31°C or 41°C. Samples were then harvested by scraping at time intervals spanning 24 hr of infection. Cells and virus were collected immediately after each harvest by centrifugation at 15,000 × *g* for 30 min at 4°C, resuspended in 0.6 ml of 10X SSC (1.5 M NaCl, 0.15 M NaOAc) / 1 M NH_4OAc per 60 mm dish, and stored at –80°C. After three freeze/thaw cycles, an equal volume of 10X SSC / 1 M NH_4OAc was added and cellular debris was removed from the samples by brief centrifugation. 20 µl of each sample was mixed with an equal volume of 0.8 M NaOH / 20 mM EDTA, denatured at 99°C for 10 min, cooled to 4°C for 1 min, and diluted with 100 µl 0.4 M NaOH / 10 mM EDTA. Using a positively charged

nylon membrane (HyBond) in a vacuum manifold (Dot Blot apparatus, Bio-Rad), equilibrated under vacuum to a flow rate of 500 µl per minute with water, 125 µl of each diluted sample (1 x 10^5 cell equivalents) was transferred to the membrane by vacuum and washed twice with excess 0.4 M NaOH / 10 mM EDTA until dry. After releasing the vacuum, the dot blot apparatus was disassembled and the membrane was neutralized in excess 0.4 M Tris pH 7.5. DNA was immobilized to the membrane by UV crosslinking (Stratalinker, Stratagene). To detect the viral DNA, RPV genomic DNA was non-radioactively labeled with digoxygenin-dUTP by random priming using a PCR DNA Labeling and Detection Kit (Roche Molecular Biochemicals). The membrane was pre-hybridized in 1X Blocking Reagent (Roche) at 68°C for 4 hr, then hybridized to digoxygenin-labeled RPV DNA (60 ng/ml) in 1X Blocking Reagent at 68°C for 13 hr in a hybridization oven. After three washes in 2X SSC / 0.1% SDS at room temperature, excess probe was removed by two stringent washes in 0.1X SSC / 0.1% SDS at 68°C for 15 min. The membrane was blocked in 1X Blocking Reagent for 30 min, then incubated with alkaline phosphatase conjugated sheep anti-digoxygenin polyclonal antibody diluted 1:50,000 in 1X Blocking solution for 30 min at room temperature. The membrane was washed in maleate buffered saline + Tween 20 solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) for 16 hr at 4°C, then incubated with CDP-Star chemi-luminescent substrate (Roche) diluted 1:500 in detection buffer (0.1 M Tris pH 9.5 / 0.1 M NaCl) for 5 min and exposed to x-ray film (X-OMAT Blue, Kodak) for visualization.

Reconstruction of RPV *sup*-2 by marker rescue. Assuming ~3 × 10^6 cells per well in 6-well plates, confluent monolayers of A549 cells were infected with RPV SPI-1 T309R at an MOI of 5 PFU per cell in 0.7 ml MEM + 10% FBS, by incubation at 37°C for 30 min. The cells were then rinsed with MEM and media was replaced with 1 ml MEM + 10% FBS. 1 µg PCR product containing the *sup-2* mutation (E9ΔH142) was pre-incubated with 4 µl Lipofectamine and 6 µl of Plus reagent (Invitrogen) diluted in 0.2 ml MEM according to the manufacturer's protocol. The cells were then transfected with this *sup-2* DNA-lipid mixture by incubation at 37°C for 3 hr. The cells were then rinsed with MEM and the media was replaced with 2 ml MEM + 10% FBS. The cells were incubated at 37°C for 24 hr, then harvested by scraping, lysed by three cycles of freeze/thaw, and titered for RPV on A549 cell monolayers. Revertant plaques were

picked and screened for temperature sensitivity (*ts*). Revertants that were *ts* were then serially plaque-purified three times on A549 cells, and amplified on CV-1 cells for DNA preparation and sequencing of E9L PCR products. RPV SPI-1 T309R recombinants containing the *sup-2* mutation were stored at –80°C for further study.

Synthesis of 5 kb PCR-based libraries for RPV and VV-WR. The entire genome of both RPV and VV-WR (vaccinia virus, WR strain) was represented in 42 and 40 overlapping 5 kb PCR products, respectively. Only non-coding DNA within the inverted terminal repeats was omitted, using the first open reading frame (RPV-UTR 001 and 184) as boundaries for the library. Sequences for the entire set of PCR primers for RPV and VV-WR are available upon request. Primer DNA sequences were based on both vaccinia (WR strain, provided by B. Moss) and rabbitpox virus (Utrecht strain, provided by M. Buller and C. Upton). Genomic sequencing data was obtained prior to publication. PCR primers were designed in pairs using Vector NTI (InforMax, Inc.) with the following optimal parameters: 26-mer length, melting temperature (T_m) each primer = 58°C ± 2, difference in T_m < 2°C, and difference in %(G+C) < 2%. Products were chosen from 4.5 to 5.5 kb, with 5 kb preferred. Each primer is entirely within a single ORF. PCR products overlap by ~ 0.5 kb (at least 0.35 kb) within the same ORF. All PCR reactions included Vent DNA polymerase at 0.032 U per µl in 1X ThermoPol Buffer (New England Biolabs), which contains 2 mM $MgSO_4$, with 0.3 µM each primer, 0.5 mM dNTPs, and 2 ng genomic wild type RPV or VV DNA per µl. For extragenic suppressor mutants, template DNA was prepared from infected CV-1 cells using the DNeasy Tissue Kit (QIAGEN) and diluted to ~120 cell equivalents per µl. PCR conditions were: 94°C for 3 min (denaturing), then 10 cycles of 94°C for 15 sec, 53°C for 30 sec (annealing), and 68°C for 5 min (extension), followed by 30 cycles with no change in denaturation or annealing conditions and 5 sec added to the extension time per cycle, and concluded with 68°C for 7 min. To remove excess primers for DNA quantitation, up to 100 µl of each PCR was purified with a 30 kDa molecular weight cutoff filter (Ultrafree-MC, Millipore) by two cycles of dilution with 350 µl H_2O and centrifugation at 5,000 × g for 5 min. DNA retained by each filter was diluted in 100 µl H_2O and stored at –20°C. The quantity of DNA in each PCR product was measured by fluorometry (TD-700 fluorometer, Turner Designs) according to the manufacturer's suggested protocol.

Western blot analysis of SPI-1, E9L, and D5R. For SPI-1 protein detection, CV-1 cell monolayers were established in 150 mm dishes, infected with an excess of each RPV mutant in 26 ml MEM with 5% FBS, and incubated at 37°C. Cells were harvested by scraping 48 hr post infection and collected by centrifugation at 300 × g for 10 min at 4°C. The cells were resuspended in 1 ml PBS per dish per sample for a final concentration of 5 × 10^4 cells per µl and stored at –20°C. For D5R and DNA polymerase protein detection, CV-1 or A549 cell monolayers were established in 100 mm dishes and infected with each RPV mutant at a multiplicity of 5 PFU per cell in 3 ml MEM with 5% FBS. After 1.5 hr incubation at 37°C, the media was replaced with 3 ml growth medium (MEM + 5% FBS) per dish to remove excess, unadsorbed virus. Cells were harvested at 6 and 9 hr post infection by scraping and were sedimented by centrifugation at 300 × g for 5 min at 4°C. Cells were then washed by resuspension with 5 ml PBS and the centrifugation was repeated. The cells were resuspended in 0.5 ml of 1 mM Tris-Cl pH 9 per dish for a final concentration equivalent to 4 × 10^4 cells per µl and subjected to three freeze/thaw cycles prior to storage at –80°C. Proteins from 1 × 10^5 (SPI-1) or 6 x 10^5 cell equivalents (E9L, D5R) were resolved on 8% (D5R) or 10% SDS-polyacrylamide gels (SPI-1, E9L) in Tris-Glycine buffer (Bloom, D. C., and Stevens, J. G., *J. Virol.*, 68:3761-3772) by electrophoresis at 100V for 1.5 hr (SPI-1) or 50V for 4.5 hr (E9L) or 8 hr (D5R). Proteins were transferred to nitrocellulose membranes by semi-dry blotting in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, pH 8.3) at 150 mA (SPI-1), 200 mA (E9L), or 250 mA (D5R) for 1 hr (SPI-1), 2 hr (E9L), or 2.5 hr (D5R). The membranes were then incubated for 16 hr at 4°C in a suspension of 5% non-fat dry milk in TTBS: 137 mM NaCl, 20 mM Tris, pH 7.6, 0.1% Tween 20. For specific detection of SPI-1, D5R, and E9L proteins, membranes were incubated with either a mouse monoclonal antibody against RPV SPI-1 diluted 1:100, a rabbit polyclonal antibody against vaccinia virus (VVwr) DNA polymerase (gift of Paula Traktman) diluted 1:2000, or a rabbit polyclonal antibody against VVwr D5R protein (Paula Traktman) diluted 1:1000 for 1 to 2 hr at room temperature. All antibodies were diluted in 5% milk/TTBS. Membranes were then washed four times with excess TTBS and incubated for 1 hr with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:10,000 (SPI-1) or goat anti-rabbit IgG diluted 1:10,000 (D5R) or 1:15,000 (E9L) in 5% milk/TTBS (SPI-1, E9L) or TTBS

alone (D5R) and washed again four times with excess TTBS. All blots used the ECL-PLUS system (Amersham) for chemiluminescent detection of secondary antibody and were exposed to x-ray film (Hyperfilm-ECL, Amersham or X-OMAT Blue, Kodak) for visualization.

Following are examples which illustrate procedures, including the best mode, for practicing the subject invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—Preparation of a 5kb PCR Library

The present inventors have developed a series of 42 PCR primer pairs with a single, common annealing temperature to generate overlapping 5kb DNA fragments that span the entire 198kb rabbitpox virus genome. Thus, in a single set of PCR reactions, it is possible to synthesize a high-resolution virtual genomic library for any given rabbitpox isolate (wild type or mutant), from a single stock of 84 oligonucleotides and commercially available DNA polymerase (such as VENT DNA polymerase).

Since most of the PCR primers were derived from the vaccinia virus genomic sequence, a virus with high homology to rabbitpox virus, this approach has been extended to develop a similar library for vaccinia virus. Data based on homology with genomic sequence data shows that these PCR primers may be used in other species of orthopoxviruses, such as smallpox (variola virus), and monkeypox viruses, with very little or no modification.

Figure 1 shows a conceptual layout of 42 PCR products present in the rabbitpox virus PCR library of the subject invention. Figures 2A and 2B are examples of 5kb PCR libraries for rabbitpox (RPV) and vaccinia (VVwr) viruses. Virtually the entire RPV and VVwr virus genomes were divided into 42 and 40 overlapping PCR products, respectively, omitting only non-coding sequence at each terminus. Each of the products shown (5µl PCR product per lane) average 5kb in length and was generated using a different PCR primer pair for each reaction. Figure 2C shows each RPV PCR product (approximately 5kb each) drawn to scale in boxes above Hind III restriction maps for RPV and VVwr.

All PCRs were run simultaneously, using the same conditions of time and temperature throughout. Establishment of such uniform conditions was achieved by choosing primers with similar characteristics of length, annealing temperature, and expected product size. This collection of PCR products represents ~195kb of genomic DNA in 5kb segments, serving the purpose of a conventional genomic library. Yet unlike randomly generated vector-borne libraries, such as those in cosmids or plasmids, no cloning is needed and the exact position of each 5kb segment with respect to the genome is predetermined by the specificity of each primer pair in the PCR for its target nucleic acid. Therefore, there is no need for secondary experiments or more time consuming methodology to determine the identity and location of each fragment in the library. Furthermore, the high yield of PCR product (several μg per 100μl PCR) is sufficient to perform the subsequent DNA mapping experiments by marker rescue without further preparation (other than removal of the PCR primers by spin filters in a centrifuge). When a conventional procedure is used, isolating 42 unique bacterial clones containing plasmids with 5kb DNA inserts followed by plasmid DNA preparations, the advantages of the subject invention becomes apparent. Both rabbitpox virus and vaccinia virus libraries are shown in Figures 2A and 2B.

The "PCR library" was used to map mutations as a substitute for cosmid libraries using the classical "marker rescue" procedure commonly used on poxviruses. Marker rescue is based on standard genetic selection that begins with infection of host cells by a virus that is unable to replicate under given "non-permissive" conditions. The infected cells are immediately transfected with individual fragments from the genomic library of a different virus that has been genetically selected for its ability to grow under these conditions. Because this is a selectable growth phenotype, the mutation responsible for allowing growth of the viral mutant must exist in at least one of the DNA fragments used for the transfection. The mutation is successfully identified because genetic recombination occurs during the infection and a single virus population emerges containing the desired mutation, called a "rescue". This first infection/transfection step maps the mutation of interest to within at least 4kb. In this example, a single 5kb fragment was identified that was able to rescue the infecting virus under the selectable growth condition used. Subsequently, using smaller PCR fragments and the same marker rescue technique, the DNA mutation responsible for the rescue of this virus was mapped

to a single open reading frame or protein. DNA within this open reading was then sequenced to reveal the exact suppressor mutation.

Figure 3 shows representative data generated using the two-step marker rescue technique. The marker rescued in this case is the ability for virus to replicate on Human Lung cells (A549). Viral replication is visualized by staining a monolayer of infected cells and looking for plaques, which are zones of clearing that represent non-adherent, dead cells. In the first step, A549 cells were infected with a mutant rabbitpox virus known for its inability to grow on these cells. These infected cells were then transfected with the 1.5kb viral PCR product shown on the right, from either a wild type or suppressor mutant genomic template. The infected-transfected cells were incubated for several days, after which all virus in the culture were harvested. The second step shown here detects the recombinant viruses generated by the first step. The two plates shown are each 35mm in diameter and covered with A549 cells. Each plate was infected with equal dilutions of virus harvested in the first step and stained with crystal violet to visualize plaques.

Transfection occurs because fragments of extracellular DNA, when combined with cationic lipids, are taken up by the cells. If a cell is infected with virus, the transfected viral DNA recombines with the infecting viral DNA, at regions of sequence homology. In this example, the viral DNA fragment used for the transfection contains the proper mutation to suppress the original mutation in the virus. Therefore, when this "suppressor mutant" DNA fragment recombines with the viral genome, the virus gains the ability to replicate and forms the plaques seen above. The 1.5kb wild type DNA fragment used in this example is from the same region as the suppressor mutation, but with a wild type genomic template. Since the sequence of this wild type fragment is identical to the infecting virus there should be no change after transfection and serves as a negative control. Therefore, cells transfected with the wild type fragment do not give the infecting virus any growth advantage, and plaques do not form, as expected.

Example 2—Orthopoxvirus Differentiation

The selectable phenotype for the mutation mapped in Example 1 was growth in a non-permissive host cell, which contributes to the understanding of mechanisms involved

in poxvirus host range. This approach may also be extended to other mutations, such as those associated with resistance to antiviral drugs.

During the primer design, selection for the position of each primer in the library was restricted to coding material, *i.e.*, within open reading frames, with special attention to maintaining the overlap of each product to within the same open reading frame, whenever possible. This restriction was imposed such that the potential use of this same set of primers for mutants or other species of orthopoxvirus was likely to succeed. In Example 1, although most of the primers were originally derived from vaccinia virus sequence, the PCR library shown in Figure 2A was generated using rabbitpox virus DNA. However, libraries for both vaccinia and rabbitpox viruses are disclosed herein and several of the primer pairs are specific for each virus. Rabbitpox is generally considered a strain of vaccinia virus due to its near identity at the nucleotide level. However, when the rabbitpox virus primer set derived from strictly vaccinia DNA sequence was applied to rabbitpox virus genomic DNA template during PCR, not all fragments were generated due to "rabbitpox virus" specific deletions, rearrangements, *etc.* However, the recent availability of unpublished rabbitpox virus genomic data has allowed the present inventors to "complete" that library by preparing PCR primers for the regions which did not amplify with the vaccinia virus-derived primer set. Subsequently, primer pairs were developed for vaccinia virus (WR strain) to complete the PCR library reagent for this species. These primer pairs were labeled "V1, V2, *etc.*" to differentiate them from the primer pairs designed for rabbitpox virus "1, 2, *etc.*". Some primer pairs with the "V" prefix are specific only to vaccinia virus WR strain and will not function to amplify products from a rabbitpox virus template. The species-specific primers that would be necessary to complete the library of any given virus would be easy to develop using the method for primer design of the present invention.

However, these natural differences between viruses allow: (1) specific primer sets to be prepared for each orthopoxvirus, including variola and monkeypox virus, for which complete genomic sequences are available, or any other species of poxvirus with very little modification; and (2) use of the rabbitpox or vaccinia primer sets with other orthopoxviruses will allow generation of PCR patterns diagnostic of that particular poxvirus. An illustrative example is shown below.

The PCR libraries of the present invention can be used as diagnostic tools for determining the species of an unknown orthopoxvirus infection. This would involve obtaining viral DNA from an infected patient, followed by PCR with sets of PCR primers developed for rabbitpox and vaccinia viruses. Figures 4A and 4B show data obtained from rabbitpox virus DNA and cowpox virus DNA, respectively. As demonstrated by comparison of the two 5kb PCR libraries, the following may be specific to certain species of orthopoxvirus: (i) number of products (out of 40-42) successfully generated; which of the 40-42 PCR products are made; and (iii) the size of the products obtained. Therefore, using the PCR primers and methods of the present invention, a potential infection (of smallpox, for example) can be readily determined and positively identified, or conversely can be ruled out, based on the PCR results. This is based on the preliminary data shown below, obtained from cowpox virus DNA compared with rabbitpox virus DNA.

The following is an exemplified protocol for using a limited set of PCR primers of the subject invention for detection of orthopox virus in a clinical setting.

Primer Pair	Poxvirus(es) distinguished
V1	VVwr
V2	VV (TianTian, WR, MVA, CPV)
V3	Var. India/Bang
V4	Ectromelia
V5	MVA
V6	Var.India/Bang, Garcia, Ectromelia
1	RPV, VV(MVA), CPV
2	RPV
8	Ectromelia
31	MVA, Monkeypox/Ectromelia, Camelpox
34	Monkeypox
37	Var.India/Garcia, Var. Bang, Monkeypox, Camelpox
39	CPV
40	Monkeypox
42	RPV
V41	VVwr, Var.India/Bang/Garcia, Ectromelia/Camelpox

V42 VVwr

14 All orthopoxviruses (positive control) 4.9kb

28 All orthopoxviruses (positive control) 4.9 to 5.1kb

(VV=Vaccinia virus; VVwr Vaccinia virus, Western Reserve strain; TianTian=Vaccinia virus, Tian Tian strain; MVA=modified vaccinia ankara strain; Var.India=Variola major virus, India strain; Var. Bang=Variola major virus, Bangladesh strain; Var. Garcia=Variola minor virus, Garcia strain; Ectromelia=Mousepox virus; CPV=Cowpox virus; RPV=Rabbitpox virus)

The 19 primer pairs listed above are prediluted with dNTPs for direct use in PCR. Refer to Table 3 for predicted sizes of each PCR product obtained with these primer pairs (expressed in kilobase pairs). Those products designated with an "x" have more than 3 mismatches between the primer and the target genomic sequence and are less likely to be successful during the PCR. Primer products in bold represent a significant size difference. For example, as indicated in Table 3, using the diagnostic methods of the subject invention, variola virus can be identified within a sample using at least one primer pair selected from the group consisting of V3, V6, 37, and V41.

Table 3

Primer Pair	RPV	VV WR	TianTian	MVA	CPV	India	Bangl.	Garcia	Monkey	Ectrom.	Camel.
V1	11.417	**5.147**	X	X	X	X	X	X	X	X	
V2	5.365	5.333	5.282	5.189	5.3?	X	X	X	X	4.867	
V3	5.349	5.342	5.341	X	5.295	**4.396**	**4.393**	5.216	5.344	X	
V4	5.034	5.047	5.026	X	5.045	4.957	4.926	4.936	5.012	4.528	5.037
V5	4.874	4.872	4.872	2.079	4.957	4.819	4.819	4.814	X	X	4.819
V6	5.439	5.411	5.411	5.391	5.559	**3.481**	**3.438**	**3.653**	5.452	6.149	5.532
V7	5.59	5.537	5.556	5.474	5.539	5.54	X	5.527	5.504	5.528	5.543
#1	5.118	X	X	2.855	5.519	X	X	X	X	X	5.507
#2	4.901	X	X	X	X	X	X	X	X	X	
#3	5.402	X	5.327	X	X	X	X	X	X	X	
#4	5.29	5.26	5.256	5.21	5.351	X	X	X	5.29	3.547	
#5	5.011	5.004	5.003	X	4.939	X	X	X	4.983	4.56	5.05
#6	4.365	4.381	4.365	X	X	4.29	4.258	4.269	4.37	4.321	
#7	5.037	5.035	5.036	X	5.129	X	X	X	5.04	5.02	5.005
#8	5.082	5.054	5.054	5.022	5.181	X	X	X	5.097	5.807	5.154
#9	5.083	5.03	5.049	4.979	5.032	5.034	5.035	5.021	4.997	5.021	5.036
#10	5.051	5.025	5.051	4.985	5.048	5.05	5.052	5.052	5.053	5.055	5.052
#11	5	4.998	4.997	4.999	4.999	4.995	4.995	4.995	4.997	4.984	4.992

Primer Pair	RPV	VV WR	TianTian	MVA	CPV	India	Bangl.	Garcia	Monkey	Ectrom.	Camel.
#12	5.077	5.077	5.076	5.074	5.151	5.05	5.056	5.056	4.885	5.123	5.067
#13	4.967	4.966	4.969	4.939	4.939	4.948	4.948	4.95	4.942	4.965	4.964
#14	4.883	4.883	4.883	4.831	4.881	4.879	4.88	4.88	4.88	4.877	4.855
#15	4.919	4.919	4.919	4.92	4.915	4.92	4.921	4.921	4.921	4.917	4.923
#16	4.879	4.879	4.879	4.88	4.88	4.88	4.88	4.898	4.881	4.879	4.879
#17	4.909	4.909	4.909	4.909	4.909	4.909	4.909	4.927	4.909	4.908	4.909
#18	5.112	5.113	5.112	5.112	5.121	5.112	5.112	5.112	5.111	5.112	5.112
#19	4.981	4.982	4.981	4.981	4.986	4.977	4.977	4.977	4.965	4.968	4.978
#20	5.095	5.049	5.05	5.053	5.047	5.037	5.037	5.037	5.046	5.05	5.048
#21	4.991	4.988	4.987	4.991	4.993	4.994	4.994	4.994	4.992	4.988	4.994
#22	4.926	4.926	4.926	4.926	4.935	4.98	4.977	4.971	4.959	4.945	4.947
#23	4.958	4.958	4.958	4.946	4.958	4.958	4.958	4.958	4.949	4.958	4.958
#24	4.994	4.994	4.994	4.994	4.994	4.994	4.994	4.994	4.993	4.994	4.994
#25	4.906	4.906	4.905	4.906	4.905	4.905	4.905	4.905	4.906	4.907	4.906
#26	4.893	4.884	4.891	4.86	X	4.857	4.857	4.857	4.891	4.887	4.868
#27	4.974	4.965	4.973	4.941	5.01	4.938	4.938	4.938	4.959	4.968	4.95
#28	4.926	4.95	4.926	4.908	4.998	4.921	4.921	4.921	4.926	4.96	5.09
#29	4.87	4.865	4.866	4.851	4.857	4.851	4.851	4.851	4.861	4.849	4.863
#30	4.974	4.974	4.971	4.975	4.971	4.971	4.971	4.971	4.965	4.977	4.968
#31	5.453	5.411	5.462	**6.399**	5.508	5.389	5.382	5.39	**4.88**	5.013	**4.689**
#32	5.355	5.4	5.4	X	5.5	5.372	5.372	5.372	X	X	5.349
#33	4.53	4.527	4.597	4.503	4.579	4.564	4.564	4.582	4.552	4.522	4.6
#34	4.99	5.004	4.982	4.882	5.017	4.934	4.91	4.934	3.877	4.971	4.532
#35	5.038	5.029	5.031	4.967	5.039	5.037	5.037	5.027	5.242	5.065	5.029
#36	5.17	5.174	5.173	X	5.184	5.146	X	5.172	X	5.157	X
#37	5.934	5.895	5.896	X	5.866	**3.945**	**3.376**	**3.989**	**3.637**	5.815	**5.193**
#38	5.708	5.691	5.694	5.531	5.756	X	5.678	5.683	5.735	5.768	5.715
#39	5.039	5.033	5.036	4.888	6.7 (3.6)	4.7	4.749	5.298	5.785	5.254	X
#40	5.007	4.976	4.973	4.959	X	4.967	4.97	5.024	**2.853**	5.05	4.745
#41	3.973	X	X	X	X	3.943	3.943	3.946	X	3.993	3.948
#42	**4.499**	X	X	X	X	X	X	X	X	X	X
#V41	X	**5.007**	X	X	X	**6.332**	**6.338**	**6.341**	X	**7.243**	**7.141**
#V42	X	**3.665**	X	X	X	X	X	X	X	X	X

VV	Vaccinia virus
VVwr	Vaccinia virus, Western Reserve strain
TianTian	Vaccinia virus, Tian Tian strain
MVA	Modified vaccinia ankara strain
Var.India	Variola major virus, India strain
Var. Bang	Variola major virus, Bangladesh strain
Var. Garcia	Variola minor virus, Garcia strain
Ectromelia	Mousepox virus
CPV	Cowpox virus
RPV	Rabbitpox virus

PCR setup:

Mix 1 (dilute DNA with primer pair)

In separate PCR tubes (thin wall, fits into thermocycler) add:

2 µl DNA sample in water

10.5 µl each primer pair (one primer pair per tube; dNTPs are included)

= 12.5µl total volume

Mix 2 (enzyme mix)

Prepare the enzyme mix in a separate tube.

For each 25µl PCR, mix: (allow one more than needed for extra volume)

0.4 µl Vent DNA Polymerase (New England Biolabs)

2.5 µl 10X ThermoPol Buffer (New England Biolabs)

9.6 µl Water

= 12.5µl total volume

Add 12.5µl enzyme mix to each DNA (Mix1) for a final volume of 25µl. For example, with one DNA sample and 19 primer pairs, make 20X of Mix 2:

20 x 0.4 µl = 8µl Vent DNA Polymerase

20 x 2.5 µl = 50µl 10X ThermoPol Buffer

20 x 9.6 µl = 192µl Water

Add 12.5µl enzyme mix to each diluted DNA sample. Final volume is 25µl.

PCR:

Seal PCR tubes well and label. Load into thermocycler.

Temperature cycling conditions for 5kb PCR to detect poxvirus:

1 cycle	94° x 3 min
10 cycles	94° x 15 sec, 48° x 30 sec, 68° x 5 min
30 cycles	94° x 15 sec, 48° x 30 sec, 68° x 5 min + 5 sec per cycle
1 cycle	68° x 7 min
1 cycle	4° hold (PCR is done)

Total PCR running time is 5 1/2 to 6 hours.

Detect PCR results by agarose gel electrophoresis.

Recommended conditions for gel preparation and electrophoresis:
0.75% agarose (high-melting) in 1X TAE buffer (Tris-Acetate-EDTA)
Use 0.5µg Ethidium bromide per ml in both the gel and the running buffer.
A normal stock solution could be 10 mg per ml (dilute 1:2000).

For best results prepare the gel as follows:
Use an Erlenmeyer flask that is at least 2.5 times the gel volume.
Add powdered agarose to 1XTAE (0.75% agarose, final) in the flask and weigh it.
Record this weight.
Boil agarose in microwave (top uncovered) until all agarose is completely melted.
(3-5 minutes)
If the flask is clean, the agarose will bubble up but not boil over.
A covered flask will boil over.

Swirl melted agarose in flask and verify its consistency by holding it up to light.
(You will need thermal protection to handle hot flask with your bare hands)
The gel has lost water during boiling and is no longer 0.75% agarose.
Place hot flask on scale and add water back to gel until it reaches original weight.

Load 1kb Ladder (NEW ENGLAND BIOLABS) for size comparison. (one well per gel)
Load 10µl PCR per well.
Run gel at 25V for 6 hours.
Gel can be monitored at will under UV after 1 hour to observe preliminary results.
Gel may also be run for more than 6 hours (depending on the length of the gel apparatus) for better clarity of size differences if necessary. Gel runs at higher voltage (50V - 100V) will run faster but will lose clarity (with smearing or "smiling") and may make size differences not as obvious.

Example 3—Infection/Transfection for Marker Rescue in Rabbitpox virus Using LIPOFECTAMINE 2000 (cationic lipid reagent)

Restore restricted host range of a SPI-1 mutant with the wild type SPI-1 gene

Marker (genetic selection): Viral plaques on the non-permissive cell line, A549.

Protocol: (2-step Marker Rescue by infection/transfection)

First, grow confluent monolayers of cells in 12-well dishes (4 cm^2 per well) (3-4 day setup of A549 cells diluted 1:6 is optimal).

Day of infection/transfection: (Warm OPTI-MEM to room temperature):

1. Pre-dilute the DNA to be transfected in a 96-well plate: 0.5 to 1μg DNA per well. (PCR product is preferred). DNA is dried in plate (overnight) to normalize volume. Add 50μl OPTI-MEM without serum. Mix.
2. Pre-dilute the lipid separately: 2μl Lipofectamine 2000 reagent per 50μl OPTI-MEM w/o serum per well. Mix.
3. Add 50μl diluted lipid to each tube and mix. Incubate 20 minutes at room temp.
4. Sonicate and dilute stock virus during this incubation, allow 0.2 ml virus per well. Recommend 400 pfu RPV SPI-1 T309R or 1200 pfu RPVΔSPI-1 per well. Use normal growth media including serum as the diluent (F-11 + 10% FBS). Add 0.2 ml diluted virus to each diluted DNA-lipid complex.
5. Remove media from the confluent cells and add complete virus/DNA/lipid mixes.
6. Incubate at 37° for 5-6 hours.

(Optional: incubation at 40° rather than 37° will reduce SPI-1 mutant "leakiness" on A549.)

7. Add 1.5ml media per well. Continue incubation at 37° (or 40°) for 5 days.
8. Harvest virus/cells with plunger of 1ml syringe on 5th day post infection. Freeze/thaw 3 times. Store virus at -80° or continue.
9. "2nd step": Sonicate and plate serial dilutions of harvested virus under non-permissive conditions (A549 cells) to detect recombinants in a standard plaque assay. Duplicates plated under permissive conditions (CV-1 cells) will indicate recombinant frequency (estimated at 1/100 to 1/1000).

Example 4—Mapping a Viral Mutation Using the 5kb PCR Library and Marker Rescue

(Estimate 1 month to map one or a group of novel mutations)

1. Plaque purify the mutant virus under selective conditions (3 rounds in 6-well dishes). Each plaque pick is diluted in 0.5ml media without serum. Freeze/thaw 3 times, sonicate, then dilute to 10^{-2}, 10^{-3}, and 10^{-4} for each round of plaque assay.

Friday (Week 1) Viral GenomicDNA preparation:

2. Inoculate 6-well plate of CV-1 cells with 100ul of plaque purified virus per well.

Monday:

3. Setup 12-well plates of A549 cells diluted 1:6 for marker rescue; 4 plates per genome. (Optionally, welled plates of other sizes can be utilized, *e.g.*, 24-well plates)

Tuesday:

4. Harvest CV-1 cells on 4th day post inf. and purify DNA using DNeasy kit (QIAGEN). Elute DNA twice with 100ul of water at final stage of purification.

5. Dilute DNA 1:5 in water for use as a template in the 5kb PCR (see protocol). ≤ 48 PCRs in 100 µl each. 2 genomes fit on one 96-well PCR plate. PCR is 5.5 hrs.

6. Pour a 1% agarose gel using the Bio-Rad 192 (multi-channel friendly) apparatus, and load 5ul of PCR product per well (dilute to 10ul with dye and water) with multipipettor. Using 51-well gel combs in 4 rows, 4 PCR libraries can be visualized per gel. Run the gels at 25V for at 6-8 hours for best picture. Use 1kb ladder for reference.

Wednesday:

7. PCR purification removes excess dNTPs and allows for quantitation of product. NUCLEO-SPIN 96 kit for vacuum filtration of PCRs in 96-well format (CLONTECH) can be used. Other suitable products for PCR cleanup in 96-well format are also available (QIAGEN), which are less expensive.

8. Quantitate DNA in 96-well plates using HOECHST dye with a standard curve (protocol). The results of the quantitation should mirror the results from the agarose gel. Expect ~10 µg DNA per PCR.

9. Calculate the volume of PCR product that equals 0.5ug DNA and distribute this volume of each PCR in a 96-well tissue culture plate (sterile) in the biological hood. Allow DNA to air dry overnight on a warm plate (~60 °C).

Thursday:

10. Follow protocol for infection/transfection of A549 cells in 12-wells (2-step MR). A significant rescue can usually be observed after the 1st step. Verify in 2nd step.

Friday (Week 2):

11. Setup 6-well plates of A549 cells for titration of infection/transfection (2nd step). Recommend at least 3 plates per genome. 6 plates is not unreasonable, to test candidates.

Tuesday:

12. Harvest infection/transfections with plunger of 1ml syringe; resuspend cells by pipetting and save only 1ml of resuspension. Freeze/thaw 3 times and sonicate.

13. Mapping: If rescue is obvious after 1st step (this is likely), then titer the harvested infection/transfection on fresh A549 cells (2nd step) in a plaque assay in 6-well dishes. If rescue was not obvious after the 1st step, continue with the mapping by pooling 10ul of each harvested infection/transfection into 6 groups and titer each group. (Recommended 10-fold dilutions for plaque assay are 10^{-3} to 10^{-5}. The mutation should be in one of the groups, unless the mutation exists in the overlapping region of two contiguous 5kb PCR products split among two groups. A successful rescue should increase the titer of virus on A549 cells at least 100-fold over background if significant.)

Friday (Week 3):

14. If using groups of harvested infection/transfections, setup 6-well plates of A549 cells for final mapping by plaque assay on Monday.

Monday:

15. Repeat this 2nd step of marker rescue with each PCR product within the successful group to map the mutation within 4kb. If one 5kb PCR product provides a rescue, then identify open reading frames within the 5kb PCR product from the genomic sequence and

make primers to amplify 3-4 overlapping PCR products (1.5kb each is recommended) within the 5kb region.

(Week 4)

16. Repeat marker rescue with the 1.5kb PCR products to identify which gene contains the mutation. Sequence the 1.5kb PCR product that provides a rescue and identify mutation.

Example 5— Host Range of RPVwt, ΔSPI-1, SPI-1 T309R, and Extragenic Suppressors

The poxvirus encoded serpin, SPI-1, is an intracellular serine protease inhibitor (serpin) that reacts to inhibit human cathepsin G *in vitro*. Rabbitpox virus (RPV) mutants deleted for the SPI-1 gene are unable to plaque on A549 cells (human lung carcinoma). Loss of host range also occurs when the ability of SPI-1 to inhibit proteases (cathepsin G) is removed by mutagenesis. The host range effects of SPI-1 suggest interactions with other cellular/viral proteins. This hypothesis was tested by first introducing a mutation into SPI-1 at the P14 residue (T309R), which is known to exist in other non-inhibitory serpins, because it restricts mobility of the reactive center loop. The SPI-1 T309R serpin is inactive as a protease inhibitor *in vitro* and leads to loss of host range in the context of RPV. The possibility of SPI-1 interactions with viral proteins was then explored by selecting for second site suppressors of the RPV SPI-1 T309R mutant that have restored plaque forming ability on A549 cells.

Both intragenic and extragenic suppressors of the SPI-1 T309R mutation have been isolated. One temperature-sensitive suppressor mutant mapped to the viral DNA polymerase (ORF E9L) by marker rescue, and was found to be thermosensitive for DNA synthesis. By developing a host range marker rescue with a 5kb PCR-based library, two other extragenic suppressors were mapped to an NTPase (ORF D5R), also required for DNA synthesis. These data unexpectedly suggest that SPI-1, to confer full host range, may interact either directly or indirectly with poxvirus proteins required for viral DNA replication.

RPV SPI-1 appears to function as a protease inhibitor *in vitro*, yet the native protease target is still unknown. Based on evidence from the bifunctional serpin SPI-3 from cowpox virus (Turner, P. and R. Moyer, *J. Virol.*, 1995, 69(10):5978-5987), it was

postulated that SPI-1, in addition to protease inhibition, may serve other functions by interacting with cellular or viral proteins at sites outside of the reactive center loop. To explore interactions of SPI-1 with poxviral proteins, we took advantage of the restricted host range phenotype of RPV SPI-1 "P14" (T309R) mutants, whose serpin conformation is compromised, to select for second-site revertants (suppressors) that restore plaque forming ability in cell culture. Using A549 cells as a non-permissive host, viruses with mutations outside of the SPI-1 gene (extragenic suppressors) have been isolated, which suggests that functions of SPI-1 other than protease inhibition may be critical in this system. Genomic mapping of sites for SPI-1 P14 mutant extragenic suppression have led to the identification of both temperature sensitive and temperature insensitive mutants in viral DNA replication enzymes.

It was hypothesized that since the P14 mutant SPI-1 (T309R) should have a slightly perturbed conformation relative to wild type, any direct interactions that may occur between SPI-1 and other viral or cellular proteins is also likely to have been affected. To pursue the hypothesis that SPI-1 may interact with other viral proteins during the infection, the reduced host range phenotype of RPV SPI-1 T309R was exploited to select for random phenotypic revertants on A549 cells, which are non-permissive for SPI-1 mutants, by plaque assay (Figure 21). Sequencing of the SPI-1 gene from plaque-purified revertant clones revealed that both intragenic and extragenic suppressors of the SPI-1 T309R mutation exist. The extragenic suppressors (Sup 1, Sup 2, and Sup 3) have a restored plaque forming ability on A549 cells similar to wild type. Normal expression of SPI-1 protein was verified by western blot, and the ability of each virus to replicate and spread in A549 cell cultures was verified by a significant increase in viral titer over the course of 3 days post infection.

The extragenic suppressor Sup 1 was mapped to a mutation in the viral DNA polymerase by rescue of its temperature sensitive phenotype. Extragenic suppressors Sup2 and Sup3 are not *ts*, but were mapped instead by rescuing the host range phenotype of RPV SPI-1 T309R infected A549 cells. Unlike a conventional temperature sensitive (*ts*) marker rescue, this "host range" (*hr*) marker rescue requires the development of individual DNA libraries for each suppressor mutant to generate the suppressor mutant DNA fragments used to map each suppressor mutation. As an alternative to the conventional approach of generating multiple cosmid libraries, a more efficient PCR-

based approach was developed in order to synthesize an effective library of 5kb products spanning the entire genome, using a series of primers with a common annealing temperature (T_m) as a reagent for each unique viral DNA template isolated. For simplification of handling, such 5kb PCR products can also be pooled together (6-7 PCR products per pool) to simulate larger DNA fragments in coarse mapping experiments (Figure 19). A 1% agarose gel loaded with 5 µl of PCR product resulting from each of the 42 different primer sets was resolved by electrophoresis for visualization (Figure 4A). The apparent consistency in the success and yield of 5kb PCR generated in a single reaction was verified by DNA quantitation, which ranged from 7-20µg total DNA for each 100µl reaction.

Extragenic suppressors were mapped using A549 cells as a non-permissive host to select for recombinant viruses. Using an infection/transfection protocol and a suppressor DNA library, viruses containing the suppressor allele were amplified under liquid media, then assayed on fresh A549 cells (Figure 3). In the example shown, only 5kb product #23 (from extragenic suppressor Sup 2 genomic DNA template) was able to rescue the reduced host range phenotype of the RPV SPI-1 T309R (*hr*) mutant. This *hr* marker rescue was repeated with smaller PCR products from *hr* suppressor DNA, and only one 1.5kb fragment that was within the open reading frame D5R was able to restore host range to the RPV SPI-1 T309R *hr* mutant. Transfection with a wild type DNA fragment covering the same region served as a negative control. DNA sequencing of the 1.5kb PCR product revealed a mutation (A330T) in the 2.5kb D5R open reading frame. The 85kDa D5R protein is an NTPase that is essential in viral DNA replication and is believed to participate in a multi-protein complex that includes the viral DNA polymerase (Evans, *et al.* 1995).

The D5R A330T mutation lies within a highly conserved region of all poxvirus D5 proteins, including members of both vertebrate and invertebrate poxvirus genera, yet the function of this domain is still unknown. Surprisingly, the remaining extragenic suppressor (Sup3) also mapped to D5, but has been identified as a promoter mutation that is immediately upstream of the D5R open reading frame. Transcriptional analyses of vaccinia virus D5R by S1a nuclease protection assays of infected cell extracts reveal that within the sequence TTCTA is the site of transcription initiation, and the suppressor mutation Sup3 is within this sequence.

It was hypothesized that the expression of D5R protein was likely to be affected by the promoter mutation in Sup3, which could also be an effect specific to A549 cells, and thus may be involved in the mechanism of restoring the wild type host range phenotype by extragenic suppression. The stability of the D5 A330T mutant was also in question, although this suppressor mutant (Sup2) is only partially temperature sensitive. Thus, to determine the steady-state levels of D5R expression, A549 and CV-1 cells were infected with each of the SPI-1 mutant and suppressor mutant viruses and compared with wild type virus at 6 and 9 hours post infection. In all samples, the expression of D5R protein was easier to detect in A549 cells than in CV-1 cells, and mutations in SPI-1 alone had no effect. The D5R promoter mutant (Sup 3) showed significantly lower levels of D5 protein in A549 cells, yet in contrast the D5 A330T mutant (Sup 2) had no effect. The phenotype of either D5 mutant (Sup2 or Sup3) in the absence of mutations in the SPI-1 gene has not yet been investigated.

Analysis of levels of other viral proteins (SPI-1 and DNA polymerase) by western blot of infected lysates confirm that the differences observed for D5 expression in the Sup3 mutant is not due to variations in total viral protein concentration. The apparent increase in D5 expression for mutants Sup1 and Sup2 in CV-1 cells is inversely correlated to the observed smaller plaque sizes for these mutants relative to Sup3, and may be attributable to a muted innate immune response to these moderately attenuated viruses at this relatively early stage of infection.

In order to determine if SPI-1 may be interacting directly with D5R, we tested the allelic specificity of the either the D5 promoter mutation or D5 A330T mutation for restoring host range to RPV SPI-1 T309R mutants and not to other SPI-1 host range mutants. We used the same host range marker rescue approach used during the extragenic suppressor mapping for this test. With this approach a "P1" mutant SPI-1 F322A or RPVΔSPI-1 was used to establish an infection in A549 cells, which were each co-transfected with PCR product containing either the D5 A330T mutation (Sup2) or the D5 promoter mutation (Sup3). Replicates transfected with either D5 PCR (surrounding codon 330) or D4-D5 PCR product (spanning the D5 promoter) obtained from the wild type RPV genomic DNA template were used as negative controls. Phenotypic revertants arising by recombination with the suppressor DNA fragments under liquid media were observed by harvesting the infected cells 5 days post infection and titering on fresh A549

cells in a plaque assay. In this second step of a two step marker rescue, equal dilutions of virus on A549 cells show at least 100-fold more plaques obtained from either D5 A330T mutant (Sup2) or D5 promoter mutant (Sup3) transfection when compared with the background signal of random revertants obtained with wild type DNA fragments. Thus, although Suppressors 2 and 3 were derived from a virus containing the SPI-1 T309R mutation, both suppressor mutations can also restore host range to other SPI-1 mutants, including RPVΔSPI-1, and is therefore not specific to the SPI-1 T309R mutation. Based on these data alone, a direct interaction between SPI-1 and D5R protein to fulfill a host range function is unlikely.

To confirm that rescued plaques were indeed recombinants containing the intended D5 mutations and were not random revertants, three rescued plaques from each of the suppressor DNA transfections and from wtDNA transfections (plated at higher concentration) were plaque purified on A549 cells. Viral DNA was isolated from each clone and used as a template for a 5kb PCR spanning ORFs D1-D5, which was later sequenced. All Sup2 transfectants contained the intended A330T mutation in D5R and all Sup 3 transfectants contained the intended D5 promoter mutation. In contrast, all of the negative controls were wild type in either the D5 ORF or the D5 promoter to within at least 400bp. The sites of the mutations in each of the revertants obtained from the negative controls are unknown and are currently being mapped.

Example 6—Selection of Primers

Examples of parameters that can be used for the design of primers of the present invention using an appropriate computer algorithm (*e.g.*, VECTOR NTI software (INFORMAX, Inc.)) are as follows:

1. 26 nucleotides in length
2. Range of T_m = 56-60 degrees C
3. delta Tm < 2 degrees
4. delta GC < 2%
5. Product size was 4.5 to 5.5kb with an emphasis on being exactly 5kb whenever possible.
6. Each primer must be entirely within a single open reading frame (ORF)
7. PCR products should overlap by about 0.5kb with a minimum of 0.35kb allowed.

8. Whenever possible, the overlap between two products should exist entirely within a single ORF.

(T_m= annealing temperature, delta T_m = difference in Tm between primers, delta GC = difference in percent G+C content, where G+C+A+T content totals 100%)

The first 4 parameters are used directly by the computer algorithm to assign a score to each primer pair designed by the computer. The higher the score, the more closely the primer pair matches the desired characteristics. The algorithm automatically excludes any regions of DNA for choosing primers that may cause problems during PCR (such as primers annealing to each other or folding back on themselves).

The only other factor that was applied during primer design was to specifically avoid having primers in the "ATI" gene of rabbitpox/vaccinia virus. This gene is known to be especially hypervariable among orthopox species, and is used in diagnostics to help identify and differentiate different clinical isolates by PCR and RFLP (restriction fragment length polymorphism) analysis, looking for size differences (Meyer, H. et al, *J Virol Methods*, 64(2):217-21, March 1997).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Claims

We claim:

1. An isolated polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or a variant of any of the foregoing.

2. An isolated polynucleotide consisting of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38,

SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or a variant of any of the foregoing.

3. A set of polymerase chain reaction (PCR) primers useful for creating a DNA library of a microorganism, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism.

4. The set of PCR primers of claim 3, wherein said at least two pairs of primers comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57,

SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or variants of any of the foregoing.

5. A method for designing a set of PCR primers for creating a DNA library of a microorganism, said method comprising providing a set of PCR primers, wherein the set of PCR primers comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism.

6. The method of claim 5, wherein the at least two pairs of primers comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID

NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or variants of any of the foregoing.

7. A set of PCR primers useful for detecting the presence of the microorganism in a sample, wherein said set comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism.

8. The set of PCR primers of claim 7, wherein said at least two pairs of primers comprise nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90,

SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or variants of any of the foregoing.

9. A method for designing a set of PCR primers for detecting the presence of the microorganism in a sample, said method comprising providing a set of PCR primers, wherein the set of PCR primers comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism.

10. The method of claim 9, wherein the at least one pair of primers comprises nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO: 67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, or variants of any of the foregoing.

11. A method of producing a DNA library of a microorganism, said method comprising:

providing a sample containing the nucleotide sequence of the microorganism;

annealing the set of PCR primers of claims 3 or 4 to the nucleotide sequence of the microorganism; and

amplifying the nucleotide sequence of the microorganism with the set of PCR primers of claims 3 or 4 in separate polymerase chain reactions, wherein each polymerase chain reaction is carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon, thereby providing a DNA library comprising the resulting amplicons.

12. A DNA library of a microorganism produced by the method according to claim 11.

13. A method of mapping a mutation of a mutant microorganism, wherein the mutation confers growth under an otherwise non-permissive condition, said method comprising:

transfecting a mutant amplicon into a host containing a microorganism lacking the mutation, wherein the mutant amplicon is from a DNA library of a mutant microorganism;

recombining the mutant amplicon with the genome of the microorganism lacking the mutation, resulting in a recombinant microorganism;

monitoring the growth of the recombinant microorganism; and

sequencing the mutant amplicon if growth of the recombinant microorganism is observed.

14. A method of mapping a mutation of a mutant microorganism, wherein the mutation confers growth under an otherwise non-permissive condition, said method comprising:

transfecting an amplicon into a host containing a mutant microorganism, wherein the amplicon is from a DNA library of a microorganism lacking the mutation;

recombining the amplicon with the genome of the mutant microorganism, resulting in a recombinant microorganism;

monitoring the growth of the recombinant microorganism; and

sequencing the mutation of the mutant microorganism if growth of the recombinant microorganism is observed.

15. A method of mapping a mutation of a mutant microorganism, wherein the mutation inhibits growth under an otherwise permissive condition, said method comprising:

transfecting a mutant amplicon into a host containing a microorganism lacking the mutation, wherein the mutant amplicon is from a DNA library of a mutant microorganism;

recombining the mutant amplicon with the genome of the microorganism lacking the mutation, resulting in a recombinant microorganism;

monitoring the growth of the recombinant microorganism; and

sequencing the mutant amplicon if growth of the recombinant microorganism is not observed.

16. A method of mapping a mutation of a mutant microorganism, wherein the mutation inhibits growth under an otherwise permissive condition, said method comprising:

transfecting an amplicon into a host containing a mutant microorganism, wherein the amplicon is from a DNA library of a microorganism lacking the mutation;

recombining the amplicon with the genome of the mutant microorganism, resulting in a recombinant microorganism;

monitoring the growth of the recombinant microorganism; and

sequencing the mutation of the mutant microorganism if growth of the recombinant microorganism is not observed.

17. A method of creating an electrophoresis pattern of at least a portion of the genomic sequence of a microorganism for the purpose of detecting the presence of the microorganism in a sample, said method comprising:

providing a sample containing the nucleotide sequence of the microorganism;

annealing the set of PCR primers of claims 7 or 8 to the nucleotide sequence of the microorganism;

amplifying the nucleotide sequence of the microorganism with the set of PCR primers of claim 3 separate polymerase chain reactions, wherein each polymerase chain reaction is

carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon;

resolving the amplicons by electrophoresis; and

obtaining an image of the amplicons resolved by electrophoresis, wherein the image is the electrophoresis pattern of at least a portion of the genetic material (*e.g.*, at least a portion of the genomic sequence) of a microorganism.

18. An electrophoresis pattern of the nucleotide sequence of a microorganism produced by the method according to claim 17.

19. A method for determining the presence of a microorganism in a sample, said method comprising:

providing a sample for testing the presence of the genomic sequence of a known microorganism;

annealing the set of PCR primers of claims 7 or 8 to any homologous polynucleotide sequence in the sample;

amplifying the sequence of any homologous polynucleotide sequence in the sample with the set of PCR primers of claims 7 or 8 in separate polymerase chain reactions, wherein each polymerase chain reaction is carried out with one pair of primers, wherein each polymerase chain reaction results in an amplicon;

resolving the amplicons by electrophoresis;

obtaining an image of the amplicons resolved by electrophoresis, wherein the image is the electrophoresis pattern of any homologous polynucleotide sequence in the sample; and

comparing the electrophoresis pattern of any homologous polynucleotide sequence in the sample with the electrophoresis pattern of at least a portion of the genetic material (*e.g.*, at least a portion of the genomic sequence) of a microorganism produced by the method according to claim 17.

20. A kit useful for creating a DNA library of a microorganism comprising:

a set of PCR primers, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a

single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism; and

at least one reagent selected from the group consisting of a buffer, a polymerase (*e.g.*, a thermostable DNA polymerase), deoxyribonucleotides, and an appropriate divalent cation.

21. The kit of claim 20, wherein said set of PCR primers is the set of PCR primers of claims 3 or 4.

22. A kit useful for creating a DNA library of a microorganism comprising:

a set of PCR primers, wherein said set comprises at least two pairs of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genetic material (*e.g.*, at least a portion of the genome) of the microorganism; and

at least one reagent selected from the group consisting of a buffer, a polymerase (*e.g.*, a thermostable DNA polymerase), deoxyribonucleotides, an appropriate divalent cation, and a positive control template.

23. The kit of claim 22, wherein said set of PCR primers is the set of PCR primers of claim 4.

24. A kit useful for detecting the presence of a microorganism in a sample comprising:

a set of PCR primers, wherein said set comprises at least one pair of primers, wherein each pair comprises one sense oligonucleotide and one antisense oligonucleotide, wherein the set of PCR primers are capable of amplifying polynucleotide sequences at a single annealing temperature, and wherein said polynucleotide sequences span at least a portion of the genome of the microorganism; and

at least one reagent selected from the group consisting of a buffer, a polymerase, deoxyribonucleotides, a magnesium salt, and a positive control template.

25. The kit of claim 24, wherein the set of PCR primers is the set of PCR primers of claims 3 or 4.

26. The set of PCR primers, or library, or electrophoresis pattern, or methods, or kits, according to any of claims 3, 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, or 24, wherein said microorganism is selected from the group consisting of virus, bacteria, yeast, and transposon.

27. The set of PCR primers, or library, or electrophoresis pattern, or methods, or kits, according to any of claims 3, 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, or 24, wherein said microorganism is orthopox.

28. The set of PCR primers, or library, or electrophoresis pattern, or methods, or kits, according to any of claims 3, 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, or 24, wherein said microorganism is selected from the group consisting of variola, vaccinia, rabbitpox, cowpox, buffalopox, monkeypox, and camelpox virus.

29. The set of PCR primers, or methods, according to any of claims 3, 5, 7, or 9, wherein said primers have a melting temperature within the range of about 55°C to about 63°C.

30. The set of PCR primers, or methods, according to any of claims 3, 5, 7, or 9, wherein each of said primers have a similar melting temperature within about 2°C, and wherein said melting temperature is within the range of about 55°C to about 63°C.

31. The set of PCR primers, or methods, according to any of claims 3, 5, 7, or 9, wherein said overlapping polynucleotide sequences are at least about 4.5 kilobases in size and within the range of about 4.5 kilobases to about 5.5 kilobases in size.

32. The method of any of claims 11, 13, 14, 15, 16, 17, or 19, wherein said amplicons are at least about 4.5 kilobases in size and within the range of about 4.5 kilobases to about 5.5 kilobases in size.

33. The method of any of claims 13, 14, 15, or 16, wherein the mutation is selected from the group consisting of host range, antibiotic resistance, drug resistance, drug dependency, and temperature resistance.

34. The method of any of claims 13, 14, 15, or 16, wherein the host is a vertebrate or invertebrate organism.

35. The method of any of claims 13, 14, 15, or 16, wherein the host is an organism selected from the group consisting of animal, plant, bacteria, archaebacteria, and fungi.

36. The method of any of claims 13, 14, 15, or 16, wherein the host is an organism selected from the group consisting of mammal, avian, amphibian, reptile, and insect.

37. The method of any of claims 13, 14, 15, or 16, wherein the host is a cell, cell culture, or tissue culture.

38. The method of any of claims 13, 14, 15, or 16, wherein the mutation causes an increase in replication of the microorganism.

39. The method of any of claims 13, 14, 15, or 16, wherein the mutation causes a decrease in replication of the microorganism.

40. The method of any of claims 13, 14, 15, or 16, wherein the sequencing is performed by the Sanger (dideoxy) method or Maxam-Gilbert method.

41. The method of any of claims 11, 17, or 19, wherein the sample is a biological sample.

42. The method of any of claims 11, 17, or 19, wherein the sample is an environmental sample.

43. The method of any of claims 11, 17, or 19, wherein the sample is selected from the group consisting of blood, serum, tissue, hair, and isolated DNA.

44. The method of any of claims 11, 17, or 19, wherein the sample is a biopsy from a lesion.

45. The method of claims 17 or 19, wherein said method further comprises recording the obtained image in a digital format or paper format.

46. The method any of claim 11, 17, or 19, wherein said method further comprises extracting DNA from the sample.

47. The method of claims 17 or 19, wherein said resolving by electrophoresis is carried out using a matrix selected from the group consisting of an agarose gel, polyacrylamide gel, and paper.

48. The method of any of claims 17, 18, or 19, wherein said electrophoresis pattern is that of Figure 2A or Figure 4A.

49. The method of any of claims 13, 14, 15, 16, or 33 wherein the mutation is selected from the group consisting of deletion, insertion, transition, and transversion.

50. The primer sets or methods of any of claims 3, 5, 7, or 9, wherein said PCR primers are within the range of about 23 to 28 bases in length.

51. The primer sets or methods of any of claims 3, 5, 7, or 9, wherein said PCR primers are 26 bases in length.

52. The primer sets, or methods, or kits, of any of claims 3, 5, 7, 9, 20, 22, or 24, wherein said amplified polynucleotide sequences are overlapping.

53. The primer sets, or methods, or kits, of any of claims 3, 5, 7, 9, 20, 22, or 24, wherein said portion of the genome comprises a region of one chromosome of the microorganism.

54. The primer sets, or methods, or kits, of any of claims 3, 5, 7, 9, 20, 22, or 24, wherein said portion of the genetic material (portion of the genome) comprises only coding sequences.

55. The primer sets, or methods, or kits, of any of claims 3, 5, 7, 9, 20, 22, or 24, wherein said portion of the genome comprises coding sequences and non-coding sequences.

5kb PCR library

(Use primers in bold for optional 15kb library)

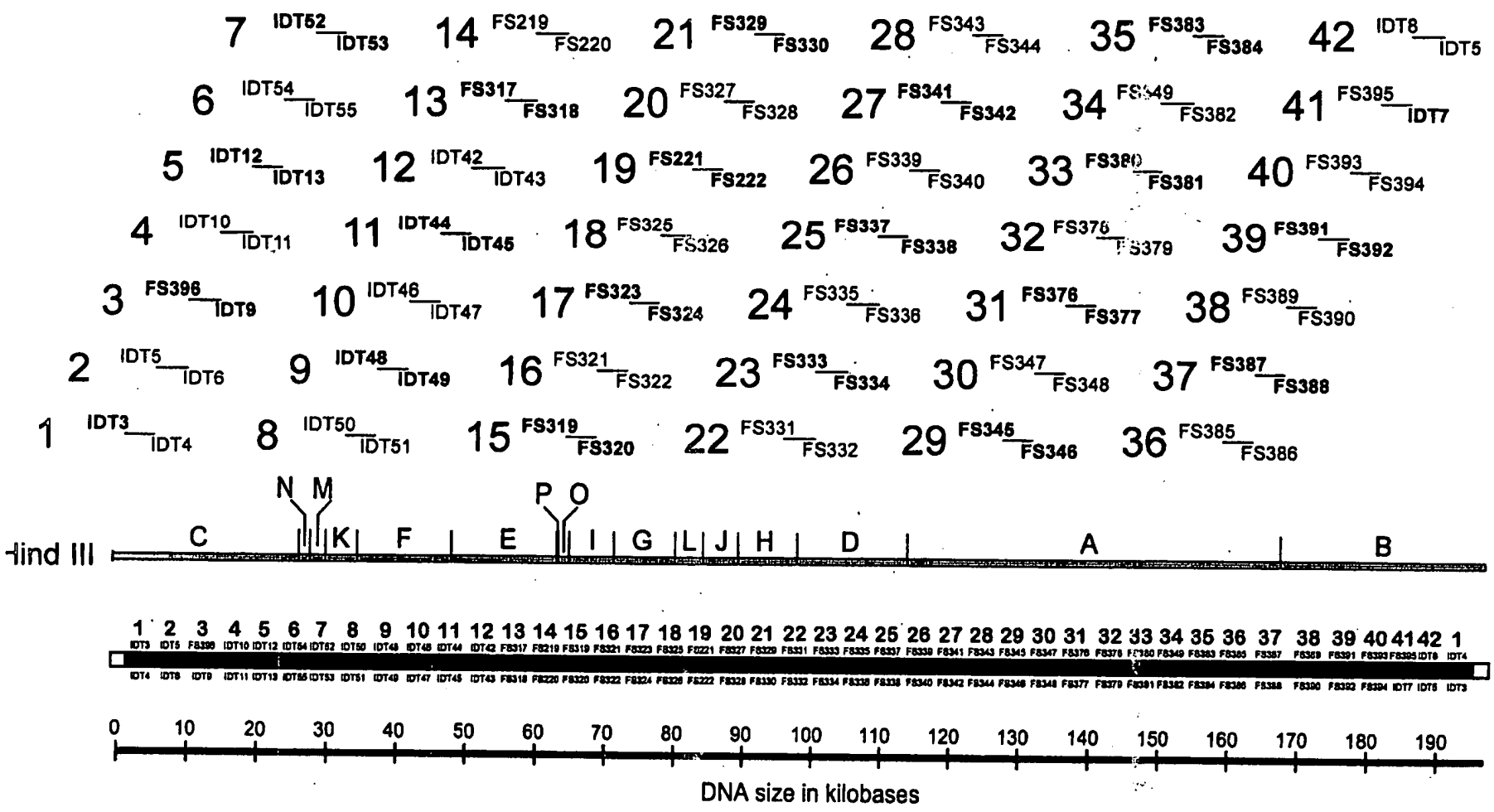


FIG. 1

FIG. 2A

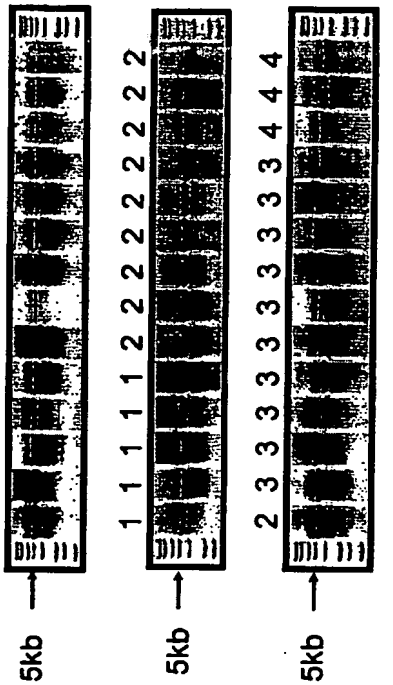


FIG. 2B

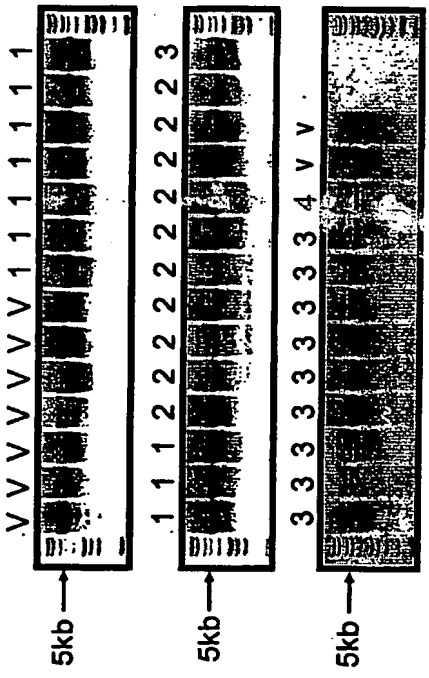
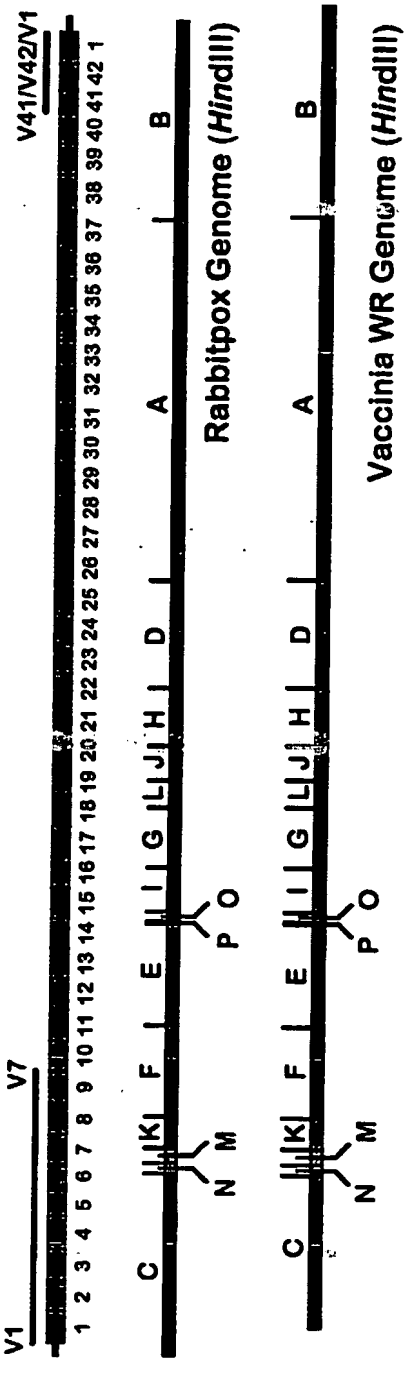


FIG. 2C



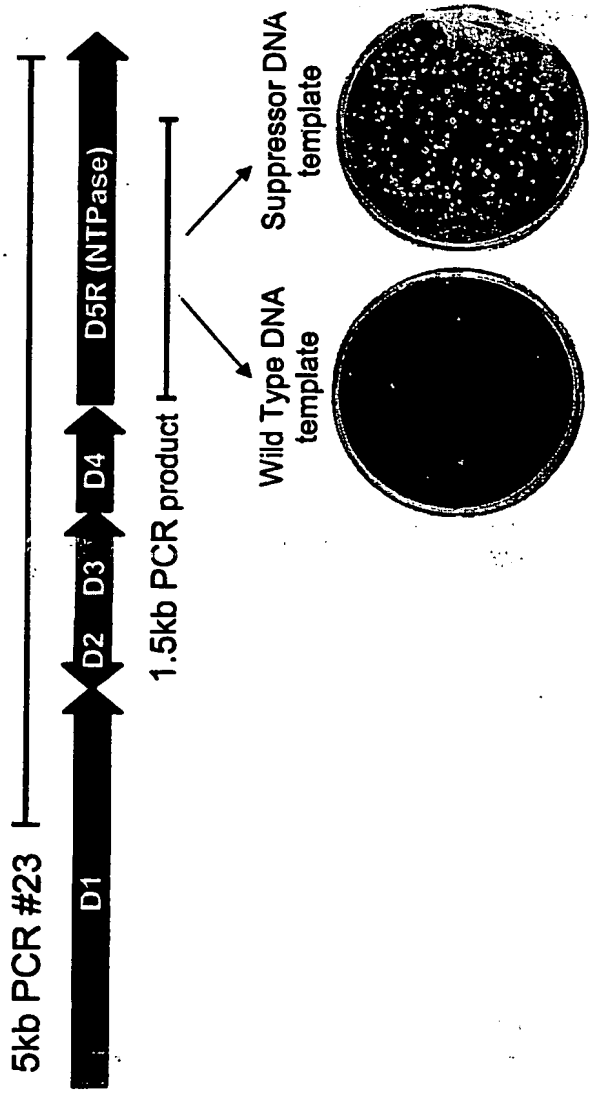

5kb PCR #23
D1
D2
D3
D4
D5R (NTPase)
1.5kb PCR product
Suppressor DNA template
Wild Type DNA template

FIG. 3

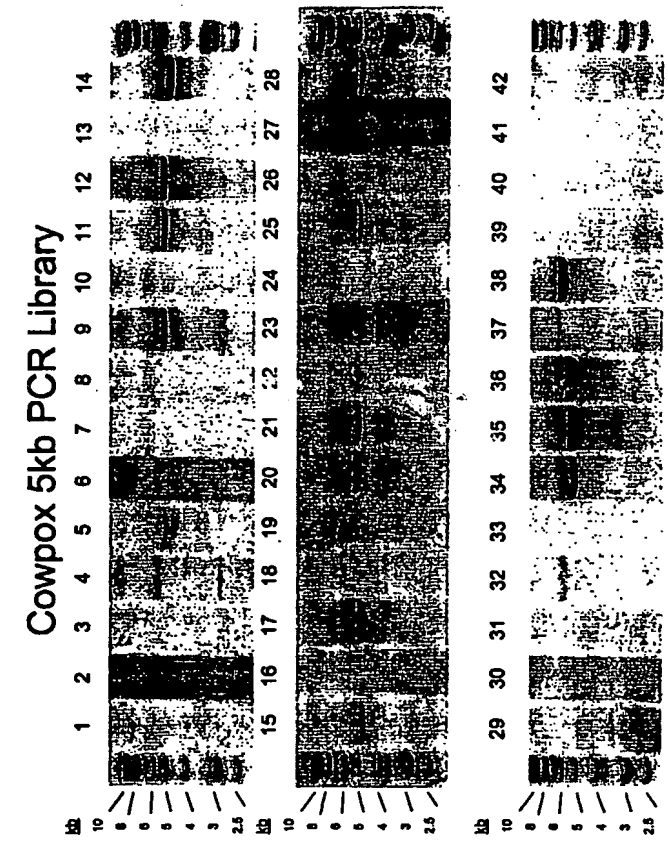


FIG. 4B

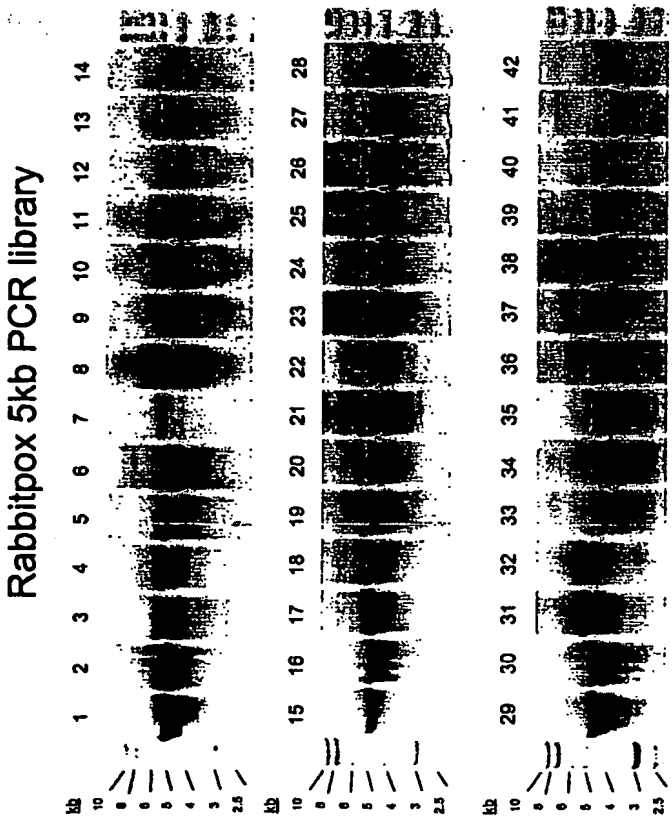


FIG. 4A

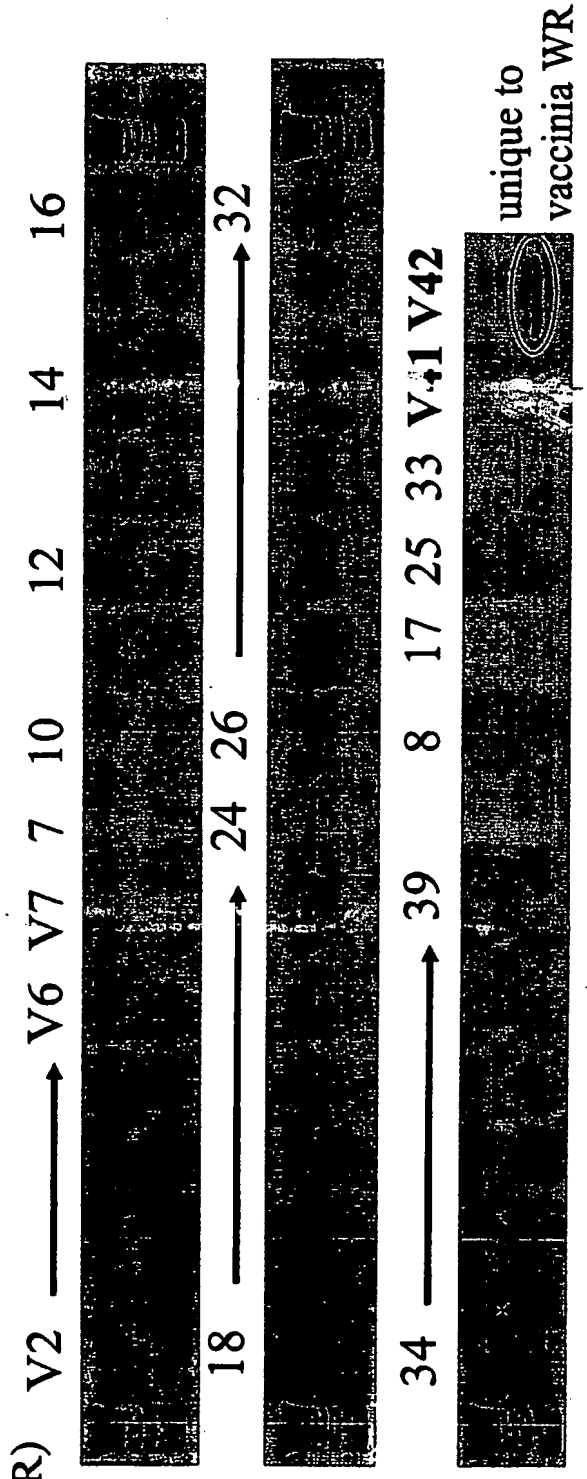

Vaccinia (WR)
PCR Library
2ng
template/µl
Tm=53°
V2
18
34
V6 V7 7 10 12 14 16
24 26 32
39 8 17 25 33 V41 V42
unique to
vaccinia WR

FIG. 5

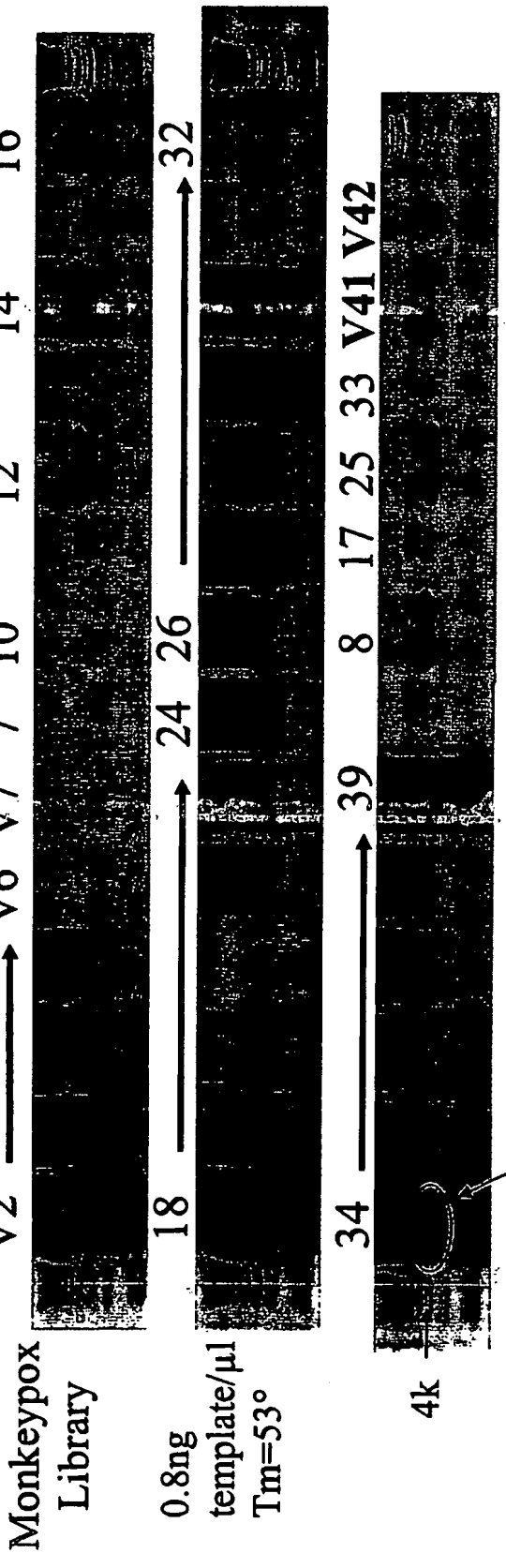

V2
V6
V7
7
10
12
14
16
18
24
26
32
34
39
8
17
25
33
V41
V42
4k
Monkeypox Library
0.8ng template/µl Tm=53°
4kb band is unique to monkeypox

FIG. 6

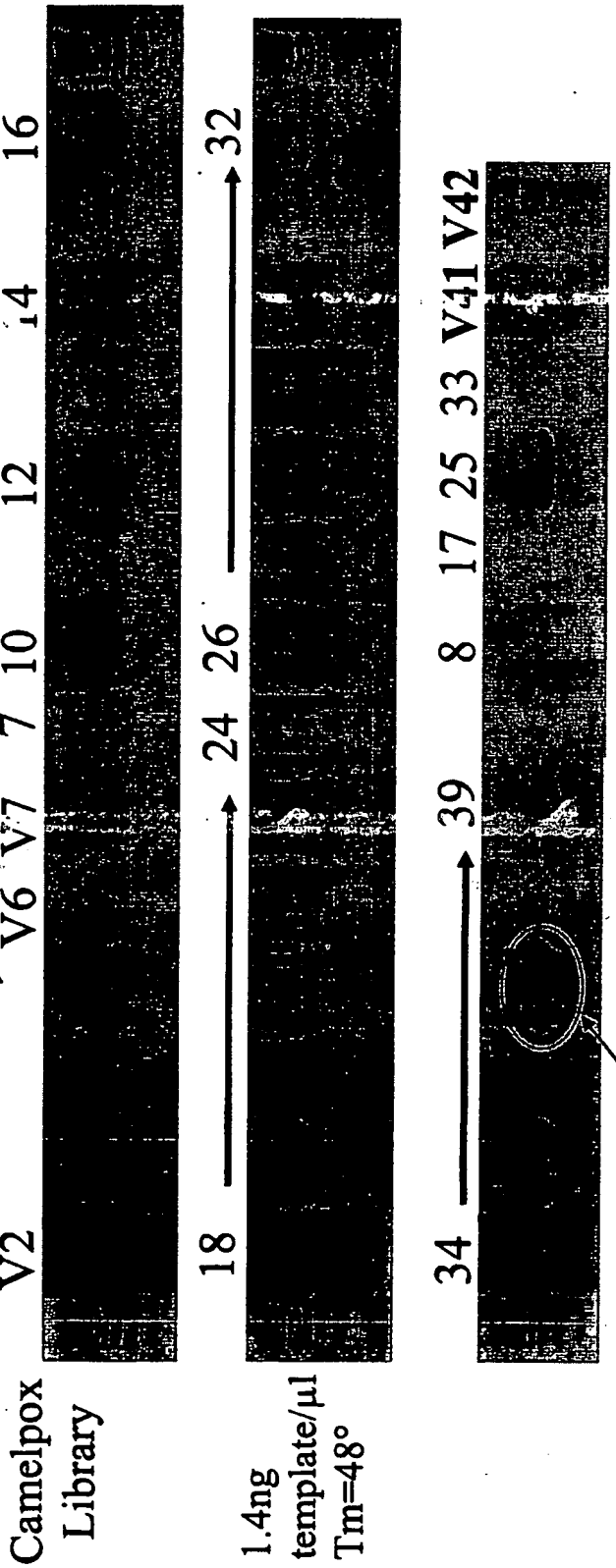

Camelpox Library
1.4ng template/µl Tm=48°
V2
V6
V7
7
10
12
4
16
18
24
26
32
34
39
8
17
25
33
V41
V42
5.2kb band is unique to camelpox

FIG. 7

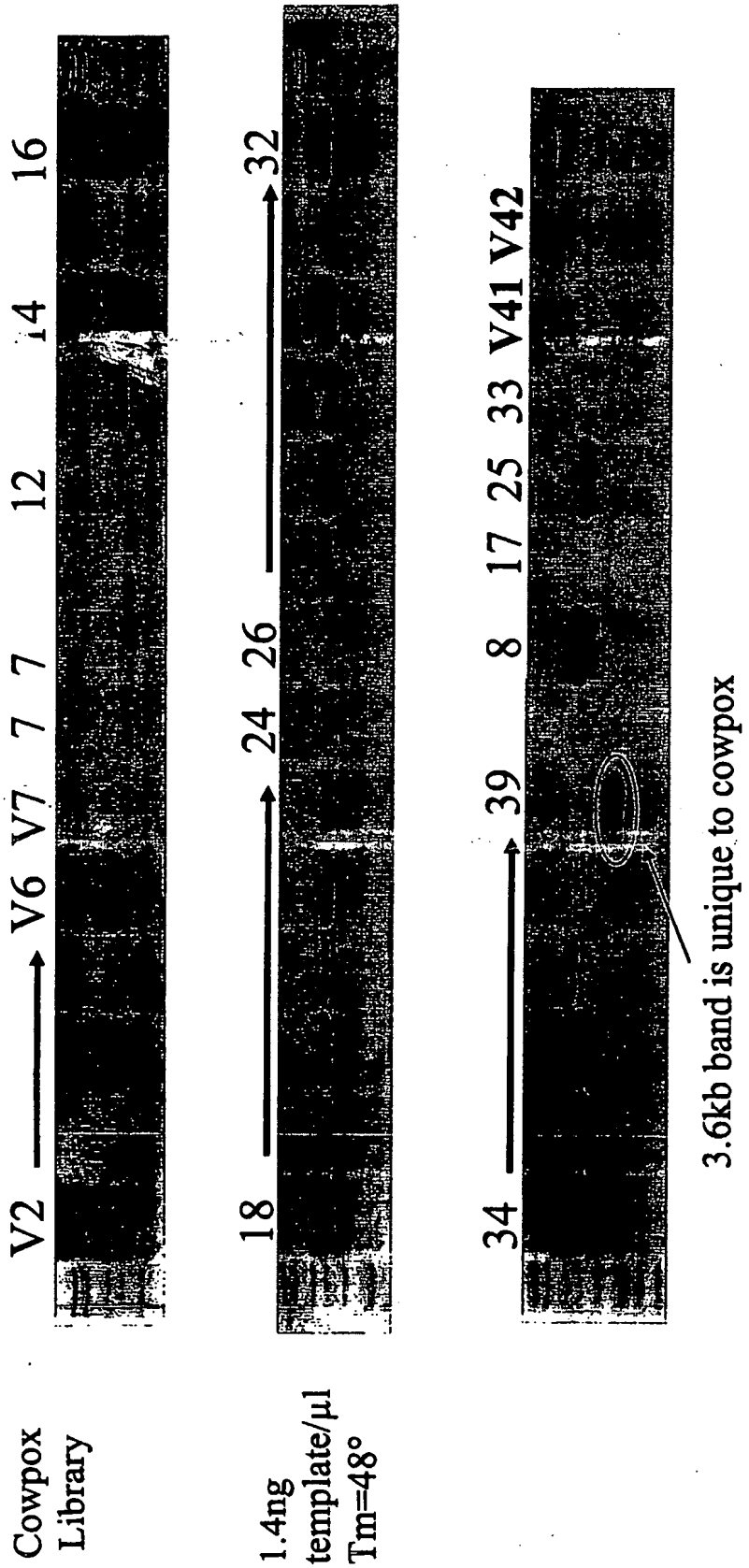

V2
V6
V7
7
7
12
4
16
18
24
26
32
34
39
8
17
25
33
V41
V42
Cowpox Library
1.4ng template/µl Tm=48°
3.6kb band is unique to cowpox

FIG. 8

FIG. 9A

Primers	VV WR	Cowpox	Monkeypox	Camelpox	India	Bangladesh	Garcia
#34	5.004	5.017	**3.877**	4.532	4.934	4.91	4.934
#35	5.029	5.039	5.242	5.029	5.037	5.037	5.027
#36	5.174	5.184	X	X	5.146	X	5.172
#37	5.895	5.866	**3.637**	**5.193**	**3.945**	**3.376**	**3.989**
#38	5.691	5.756	5.735	5.715	X	5.678	5.683
#39	5.033	**3.5**	5.785	X	4.7	4.749	5.298

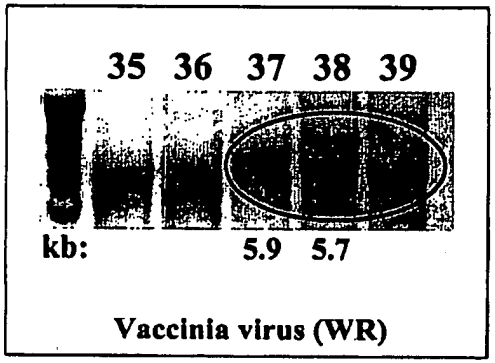


FIG. 9B

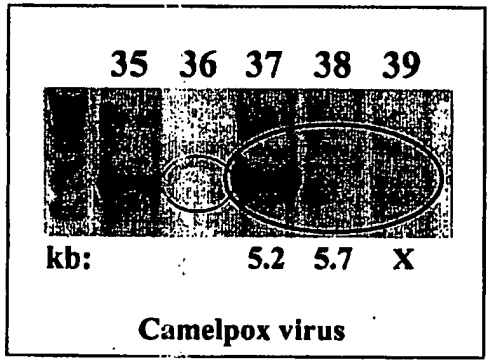


FIG. 9C

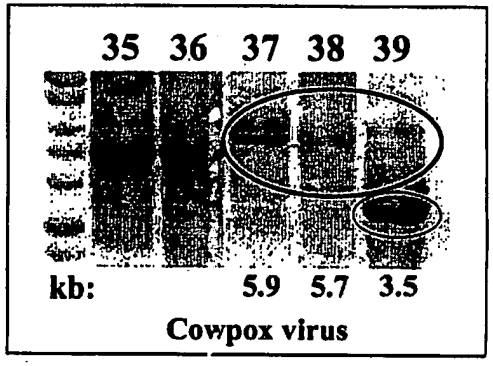


FIG. 9D

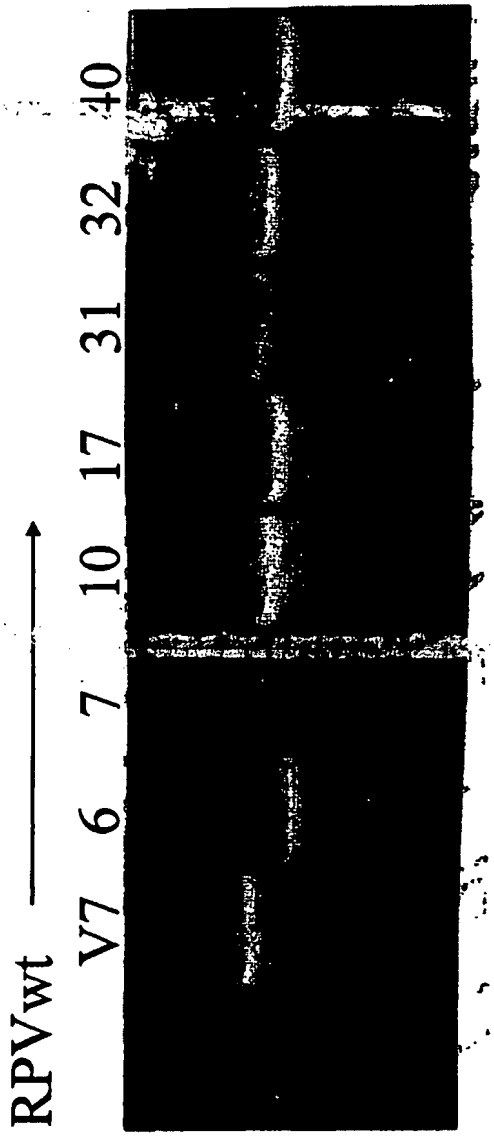


FIG. 10

5kb PCR Libraries

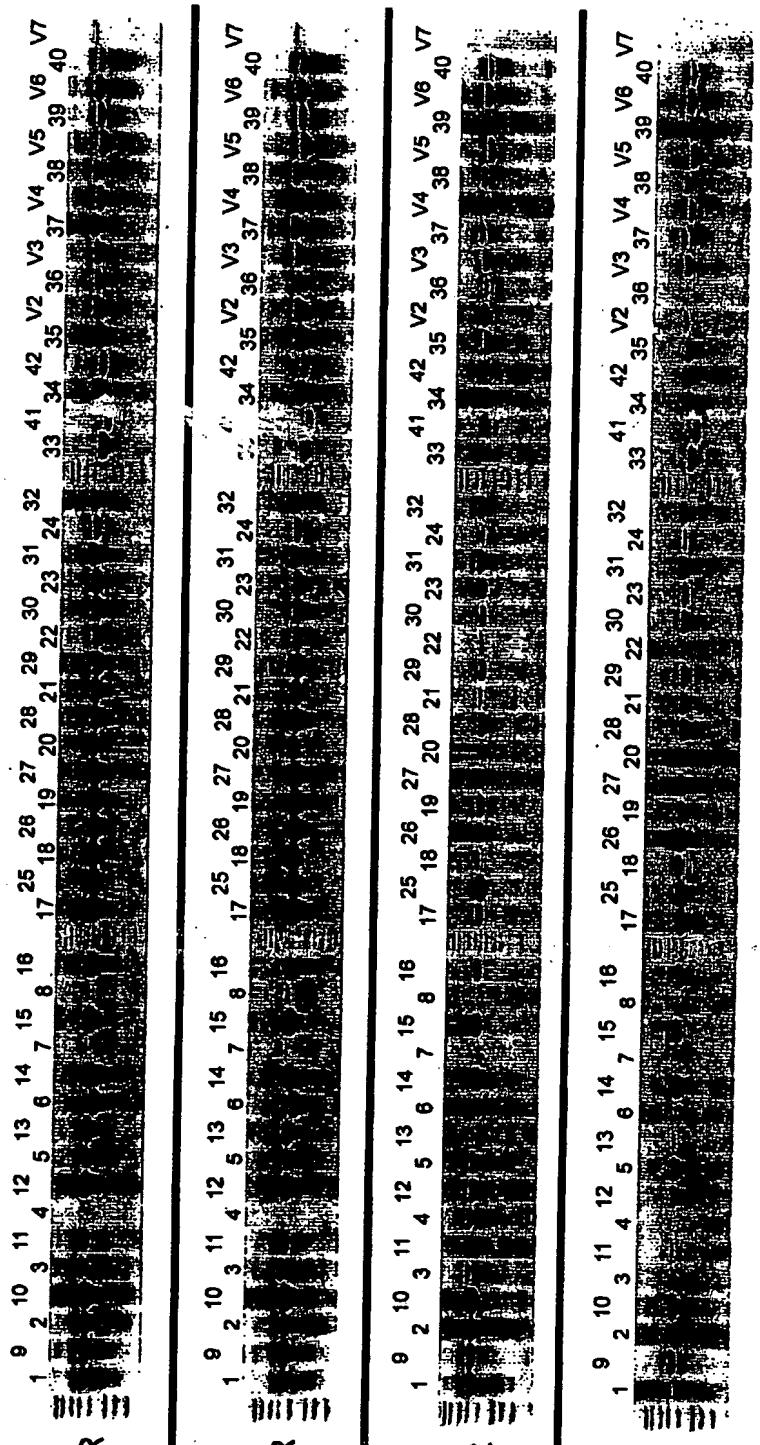


FIG. 11A

FIG. 11B

FIG. 11C

FIG. 11D

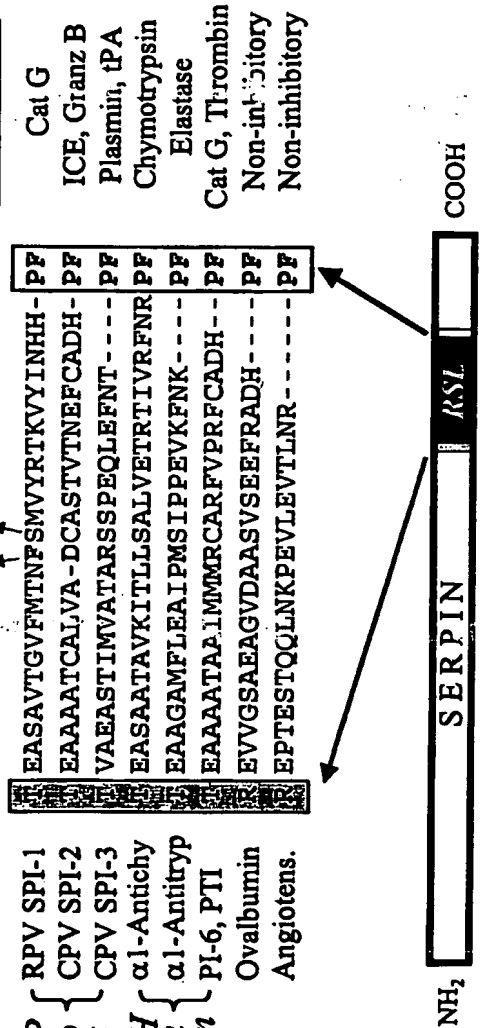

P14
P1 P1Õ
Protease Targets
Cat G
ICE, Granz B
Plasmin, tPA
Chymotrypsin
Elastase
Cat G, Thrombin
Non-inhibitory
Non-inhibitory
EASAVTGVFMTNFSMVYRTKVYINHH-PF
EAAAATCALVA-DCASTVTNEFCADH-PF
VAEASTIMVATARSSPEQLEFNT----PF
EASAATAVKITLLSALVETRTIVRFNR PF
EAAGAMFLEAIPMSIPPEVKFNK----PF
EAAAATAAIMMMRCARFVPRFCADH--PF
EVVGSAEAGVDAASVSEEFRADH----PF
EPTESTQQLNKPEVLEVTLNR------PF
P
o
x
H
u
m
RPV SPI-1
CPV SPI-2
CPV SPI-3
α1-Antichy
α1-Antitryp
PI-6, PTI
Ovalbumin
Angiotens.
NH2
SERPIN
RSL
COOH

FIG. 12

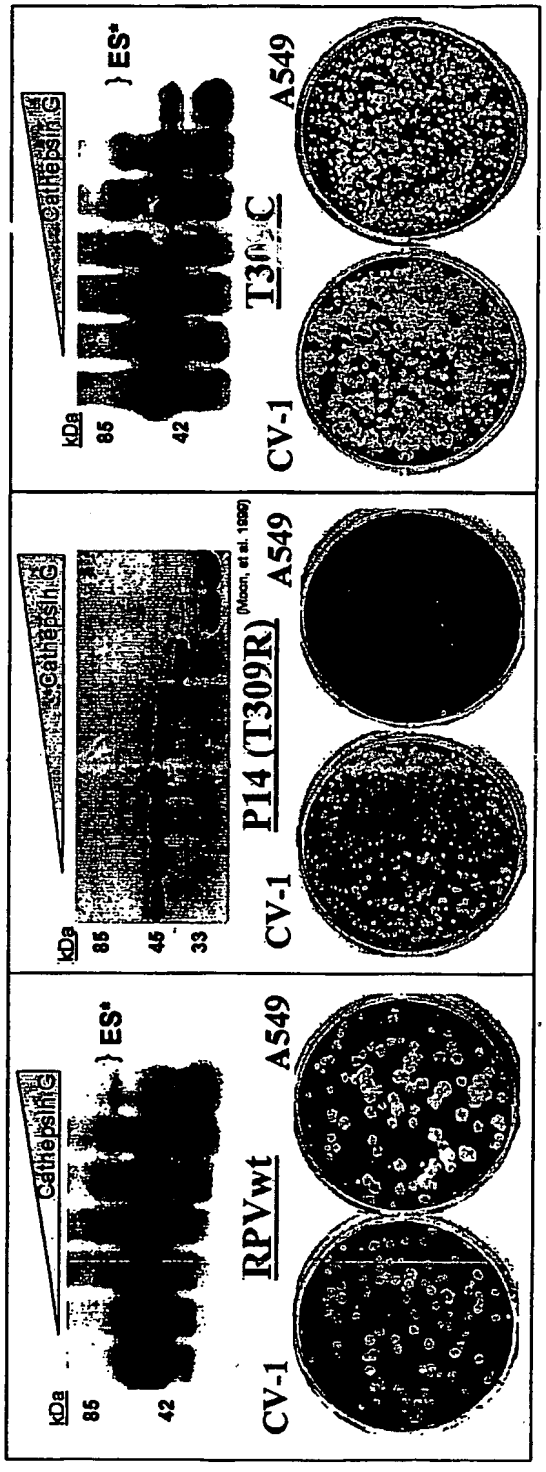


FIG. 13A FIG. 13B FIG. 13C

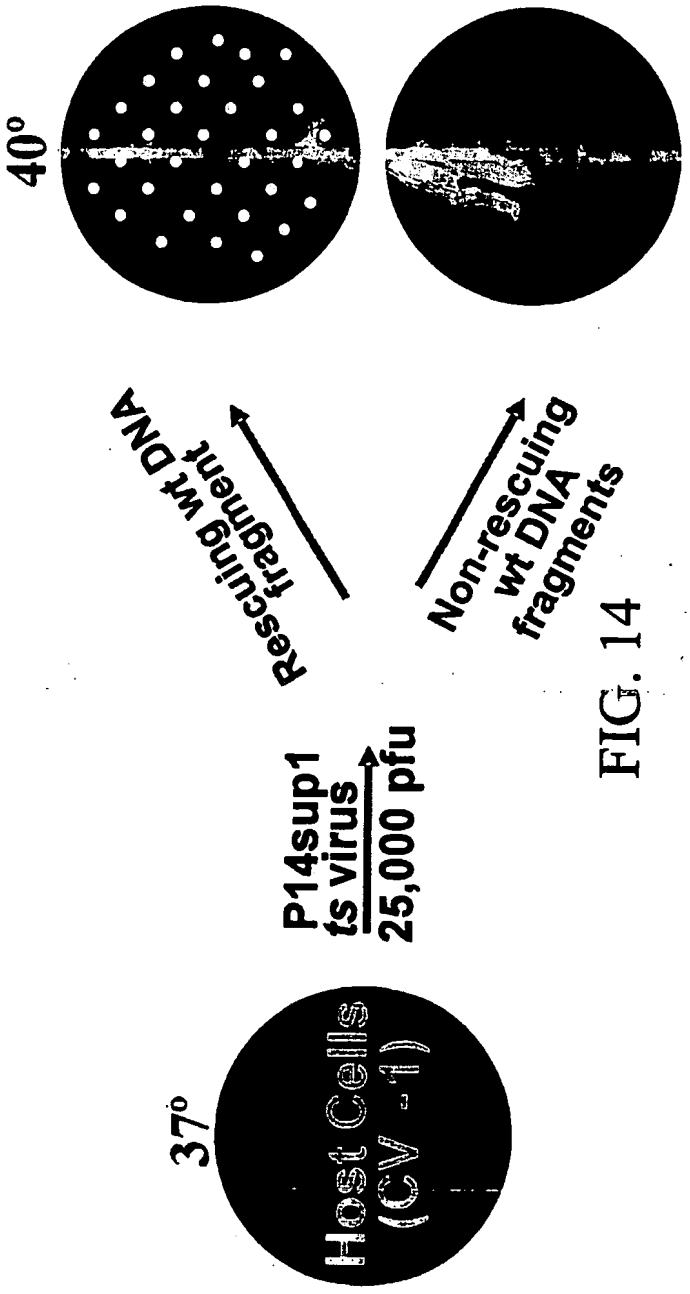

40°
Rescuing wt DNA fragment
Non-rescuing wt DNA fragments
P14sup1 ts virus 25,000 pfu
37°
Host Cells (CV-1)

FIG. 14

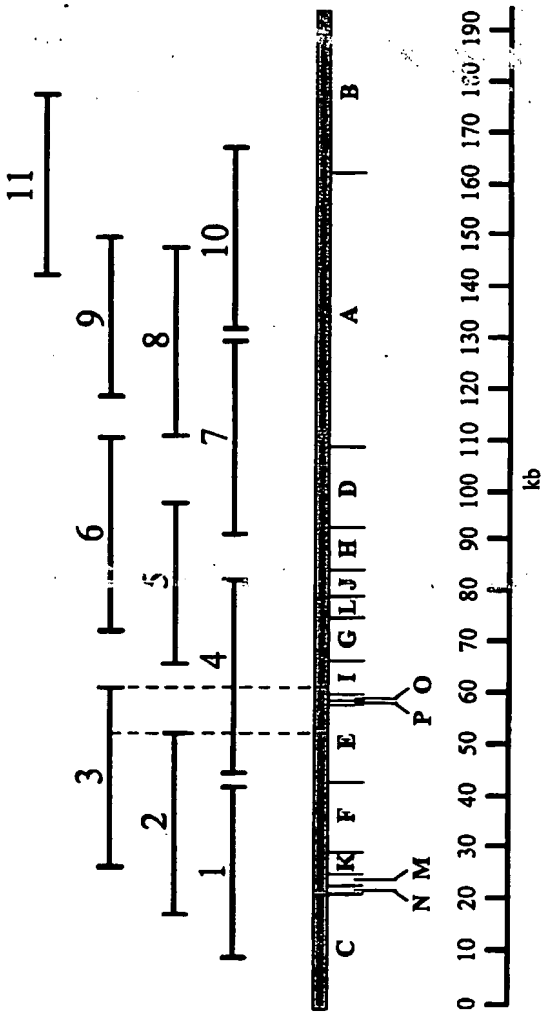

11
9
8
10
6
5
7
4
3
2
1
B
A
D
H
J
L
G
I
E
F
K
C
N
M
P
O
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
kb

FIG. 15

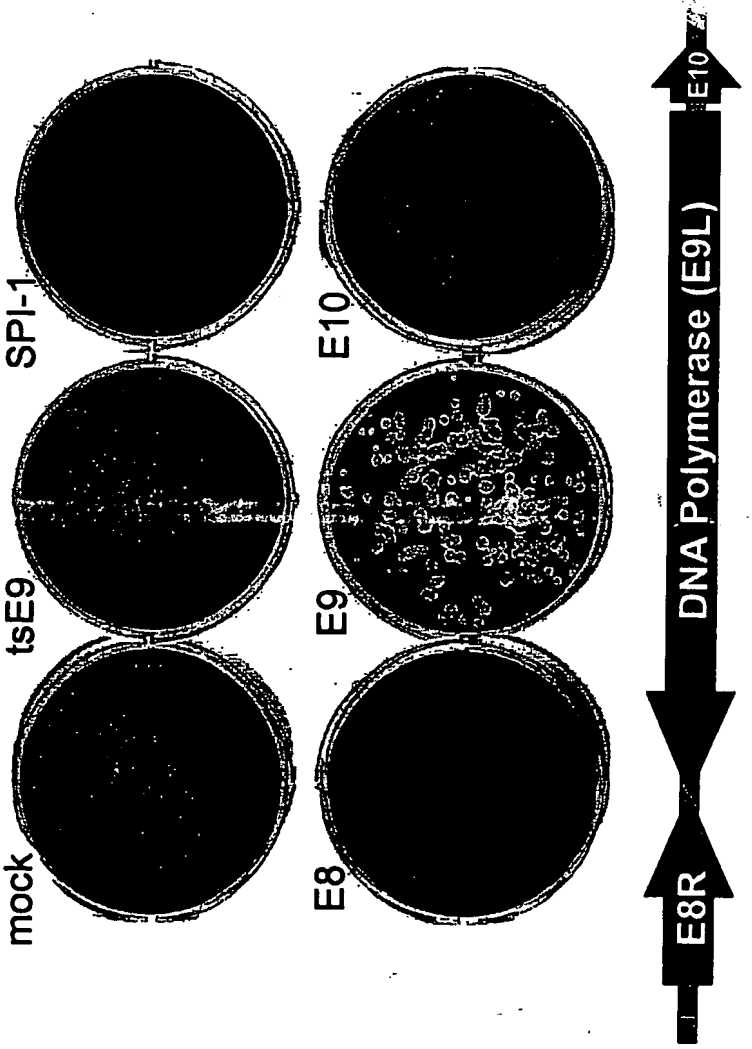


FIG. 16

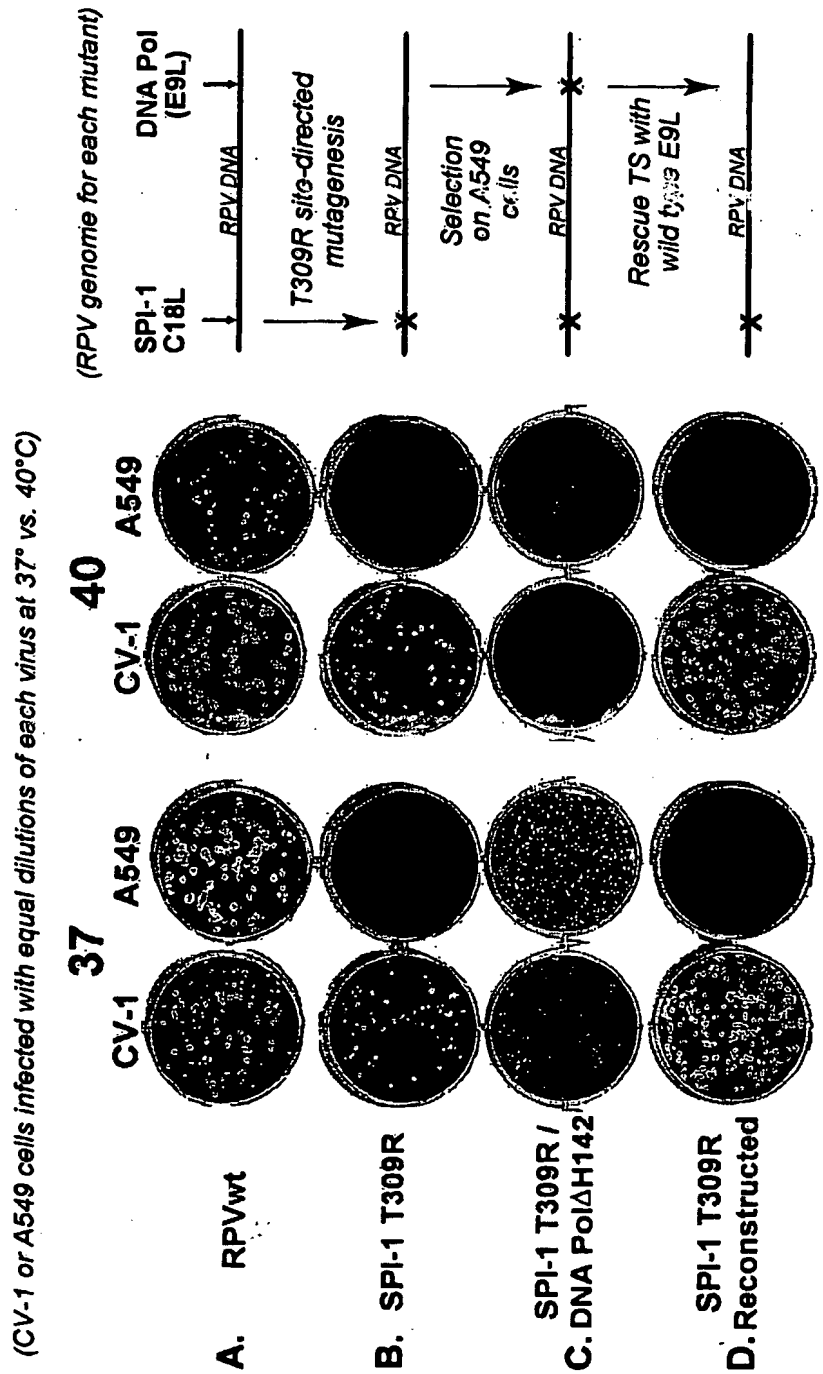

(CV-1 or A549 cells infected with equal dilutions of each virus at 37° vs. 40°C)
37
40
CV-1
A549
CV-1
A549
A. RPVwt
B. SPI-1 T309R
C. SPI-1 T309R / DNA PolΔH142
D. SPI-1 T309R Reconstructed
(RPV genome for each mutant)
SPI-1 C18L
DNA Pol (E9L)
RPV DNA
T309R site-directed mutagenesis
Selection on A549 cells
Rescue TS with wild type E9L

FIG. 17

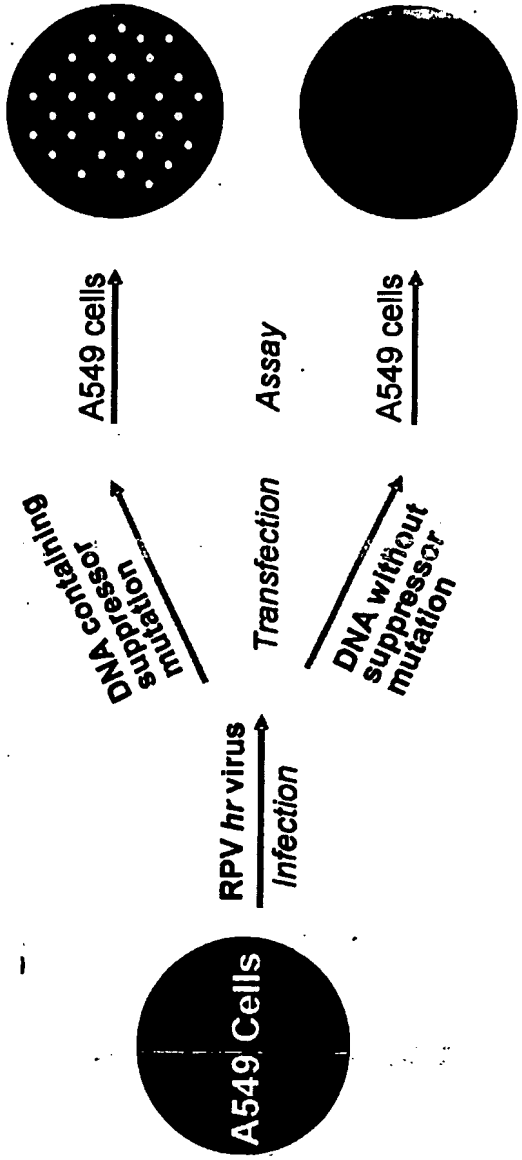

A549 Cells
RPV hr virus
Infection
DNA containing suppressor mutation
Transfection
DNA without suppressor mutation
A549 cells
Assay
A549 cells

FIG. 18

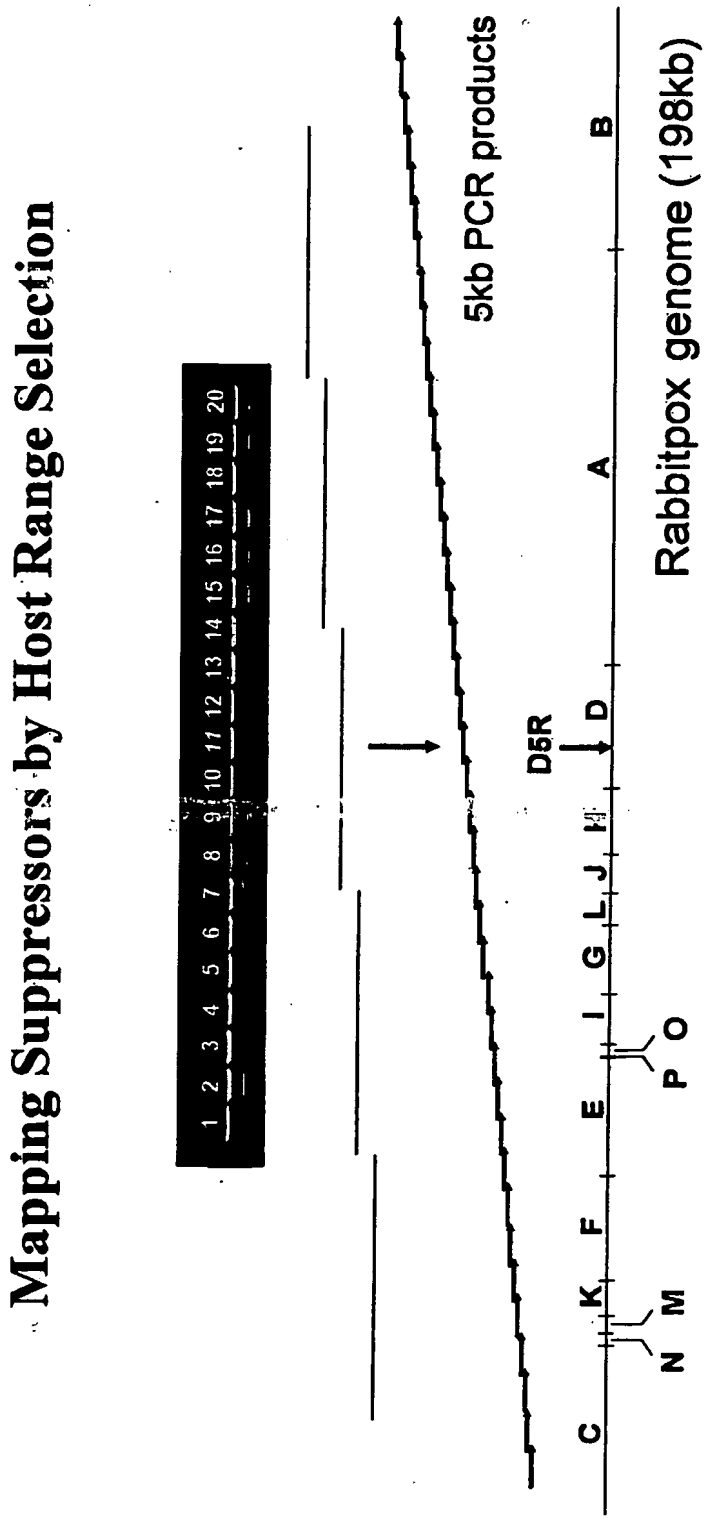

Mapping Suppressors by Host Range Selection
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5kb PCR products
D5R
Rabbitpox genome (198kb)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

FIG. 19

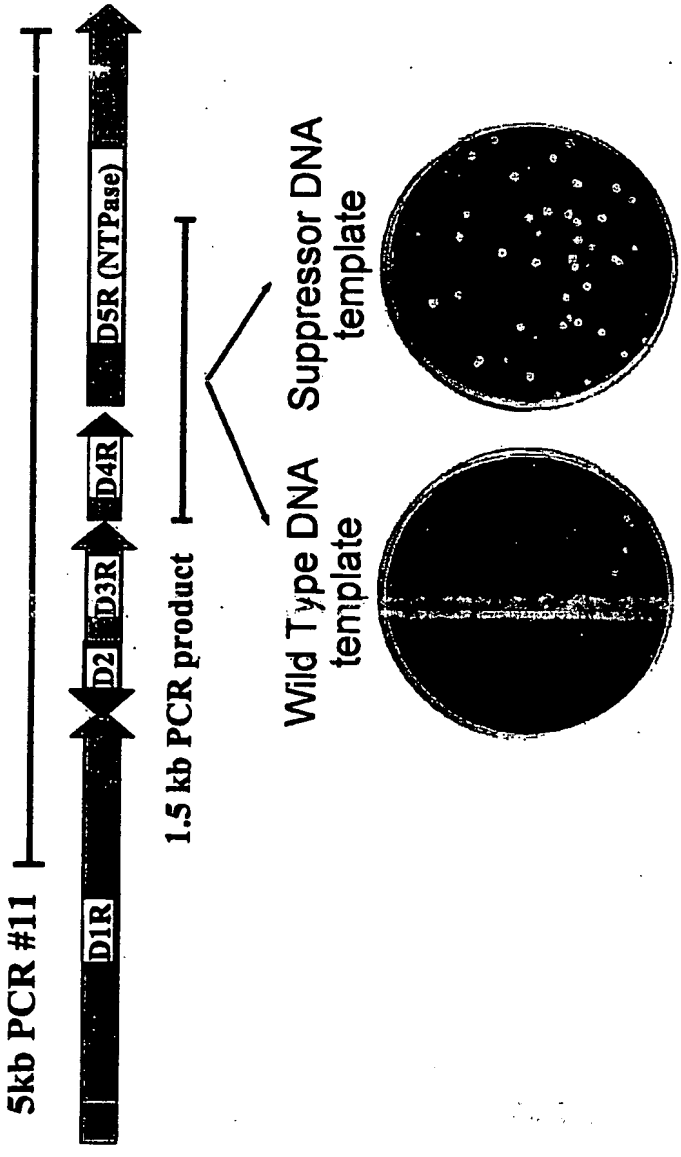

5kb PCR #11
D1R
D2
D3R
D4R
D5R (NTPase)
1.5 kb PCR product
Wild Type DNA template
Suppressor DNA template

FIG. 20

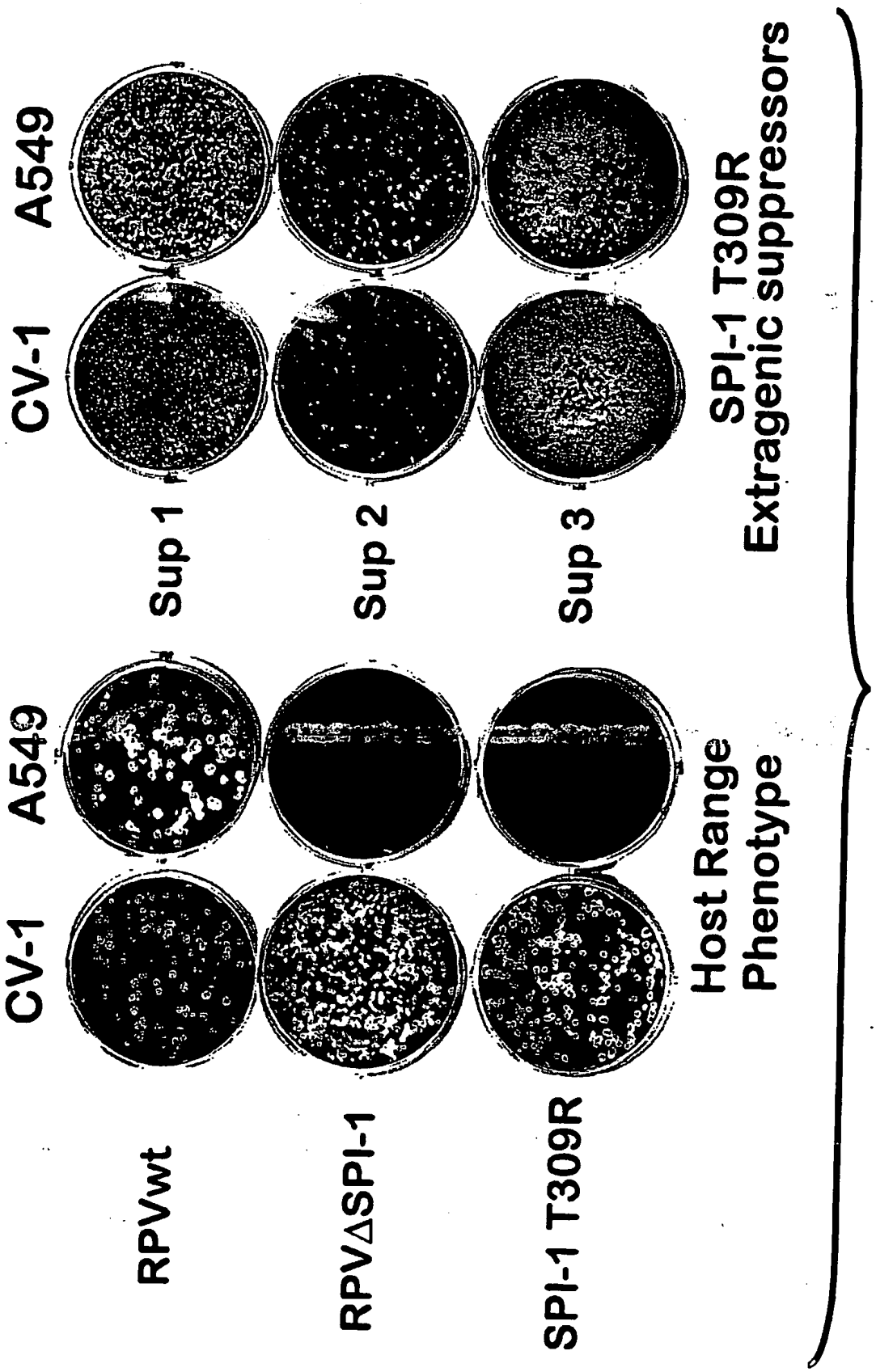

CV-1
A549
CV-1
A549
Sup 1
Sup 2
Sup 3
SPI-1 T309R
Extragenic suppressors
RPVwt
RPVΔSPI-1
SPI-1 T309R
Host Range
Phenotype

FIG. 21

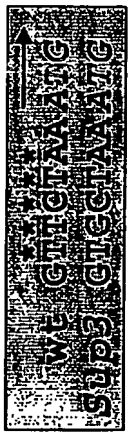


FIG. 22A

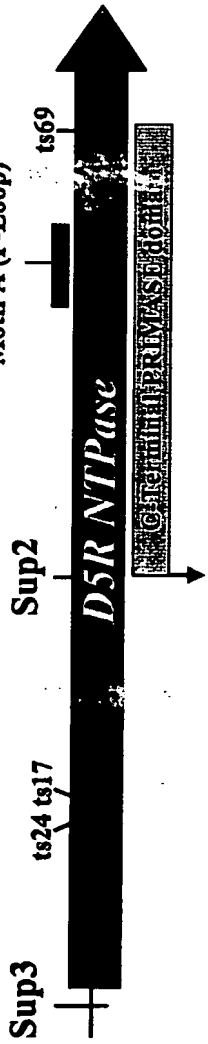


FIG. 22B

```
SUP2                                  T
RPV  323 GNKLFNIAQRILDTNSVLLTERGD 346
MYX      GNKLFTISQIIMDSNVIHLTDRGE
YLDV     GNKLFTISQLIMDANVIRLTERGD
MCV      GNRLFTIAQRILDANVINLTERGD
FPV      GNKLFSISQQIIDLNVINVSDRGD
                    helix
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FIG. 22C

SEQUENCE LISTING

<110> University of Florida Research Foundation, Inc.

<120> Materials and Methods for Producing Virtual Genomic Libraries

<130> UF-359XC1 PCT

<150> 60/462,204
<151> 2003-04-11

<160> 98

<170> PatentIn version 3.2

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<210> 2
<211> 26
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<210> 3
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<220>

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<210> 4
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<220>

<223> oligonucleotide primer IDT 6 of primer pair #2 and #V42

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<210> 6
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<210> 7
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<212> DNA
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<220>
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tcacgtaatt ctggattatt ggca 24

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<210> 9
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<212> DNA
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<400> 9
gtgtttgaag agctcgttct catca 25

<210> 10
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<220>
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<400> 10
gccataataa ttgatgttcc gcc 23

<210> 11
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<220>
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gtttttcaca gttgctcaaa aacgat 26

<210> 12
<211> 26
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<220>
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<400> 12
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<210> 13
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<220>
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<400> 13
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<210> 14
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<212> DNA
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<400> 14
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<220>
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<220>
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<210> 21
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<210> 24

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<210> 29
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<400> 29
aaatagtcac gcaattcatt ttcggg 26

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<400> 30
tgcttttgat ggtaatttct ggtgcc 26

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ggcataatcc ggatgttgtg tagtac 26

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<400> 32
gtagcgtttg ttccgttata gacacc 26

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<400> 33
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<210> 34
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<400> 34
tatcgttcgt gagaaatatc tttgcc 26

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<400> 35
actcgtatat tcctccttgt caatgc 26

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<400> 36
ttatggcagg tgagatgttt gttaga 26

<210> 37
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<212> DNA
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<400> 37
cattaaggcg ttgatgcaat tgacga 26

<210> 38
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<220>
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<400> 38
tggttctcca taatcatcat caaccg 26

<210> 39
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ttcacatgta ctttatgctg aggacc 26

<210> 40
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<400> 40
catgagaaga cccaagtcga taaagt 26

<210> 41
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<400> 41
tcattgtaaa ggaatggggt atggaa 26

<210> 42
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<400> 42
agaacagaat attgacgcgg atgatc 26

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<400> 43

ttttggaaa tgggaatacc gatgtg 26

<210> 44
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<400> 44
aataatggat agcaaactgc cagtcg 26

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cgaggataaa ctatcggatg tgggac 26

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<400> 46
ttggaaagta gtcaacggaa gagtga 26

<210> 47
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<400> 47
gacagaacct tgtgtcattg gaagac 26

<210> 48
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<220>
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<400> 48
tcagcagtag ctggatctag agaaaa 26

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<400> 49
gcatggcgtg ttttattaat caatcg 26

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<400> 50
aaactccgtt tgatgtggaa gataca 26

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<400> 51
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tatccaaccg ctacaaccaa ctattc 26

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aggtgtagga gtacatacag tggcca 26

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<400> 54
tgttcatctc gaatagccag agctac 26

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<400> 55
taatttggag gcctccgtag aactag 26

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<211> 26
<212> DNA
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<220>
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<400> 56
tatcgactca gttaatgctg ggaaaa 26

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<400> 57
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<400> 58
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<400> 60
gatagacaac ttctggaggc atatcg 26

<210> 61
<211> 26
<212> DNA
<213> artificial

<220>
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<400> 61
ctccagatta ctcacctata atcgcg 26

<210> 62
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 377 of primer pair #31

<400> 62
gaacttgaac tcagtcgtat gtggct 26

<210> 63
<211> 26
<212> DNA

<213> artificial

<220>
<223> oligonucleotide primer FS 378 of primer pair #32

<400> 63
tttcggcatt ggtttcatta ttacgt 26

<210> 64
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 379 of primer pair #32

<400> 64
taaaatggcc cttgttaaac attgga 26

<210> 65
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 380 of primer pair #33

<400> 65
caaacacgat ttaaaatcaa accacg 26

<210> 66
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 381 of primer pair #33

<400> 66
aatgaacgga tttgacttgc tacaaa 26

<210> 67
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 349 of primer pair #34

<400> 67
atgatgctgg tacctcttat cacggt 26

<210> 68
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<212> DNA
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<220>
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<400> 68
ctagacgaac ccctcagaca aacaac 26

<210> 69
<211> 26
<212> DNA
<213> artificial

<220>
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<400> 69
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<210> 70
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer FS 384 of primer pair #35

<400> 70
gcaaccatcc aaagattttc atctct 26

<210> 71
<211> 26
<212> DNA
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<220>
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<400> 71
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<210> 72
<211> 26
<212> DNA
<213> artificial

<220>
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<400> 72
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<210> 73

<211> 26
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<400> 73
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<210> 74
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<400> 74
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<210> 75
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<220>
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<210> 77
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<210> 79
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<400> 79
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ataaagatcg ttaattgtca gcatgt 26

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gaatgtgata agatcgttgg tggatc 26

<210> 82
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<400> 82
acatcatata ctcgaggacg gcatta 26

<210> 83
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ggttactttg aaggacgtac accact 26

<210> 84
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<400> 84
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<210> 85

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<210> 86
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<400> 87

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<210> 88
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<210> 89
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<220>
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<400> 89
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<210> 90
<211> 26
<212> DNA
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<220>
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<400> 90
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<210> 91
<211> 26
<212> DNA
<213> artificial

<220>
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<400> 91
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<210> 92
<211> 26
<212> DNA
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<220>
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<210> 93
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<212> DNA
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<210> 95
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<210> 96
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<220>
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<400> 96
aattactatc ccacttttat ccggca 26

<210> 97
<211> 26
<212> DNA
<213> artificial

<220>
<223> oligonucleotide primer IDT 395 of primer pair #V41

<400> 97
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<210> 98
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<400> 98
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